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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Paul C. Anderson *et al.*

Serial No.: 09/732,439

Filed: December 7, 2000

For: TRANSGENIC MAIZE WITH
INCREASED PROLINE CONTENT (AS
AMENDED)



Group Art Unit: 1638

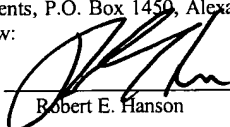
Examiner: Cynthia E. Collins

Atty. Dkt. No.: DEKM:184USD1

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March 12, 2004
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Robert E. Hanson

BRIEF ON APPEAL

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TABLE OF CONTENTS



I.	REAL PARTIES IN INTEREST	3
II.	RELATED APPEALS AND INTERFERENCES.....	3
III.	STATUS OF THE CLAIMS	3
IV.	STATUS OF AMENDMENTS	3
V.	SUMMARY OF THE INVENTION	4
VI.	ISSUES ON APPEAL	4
VII.	GROUPING OF THE CLAIMS.....	4
VIII.	SUMMARY OF THE ARGUMENT	5
IX.	ARGUMENT.....	6
A.	The Written Description Rejection Was Improperly Maintained.....	6
B.	The Enablement Rejection Was Improperly Maintained.....	8
C.	The Indefiniteness Rejections Were Not Properly Maintained	10
D.	The Anticipation Rejection Was Not Properly Maintained	11
E.	The Obviousness Rejection Was Not Properly Maintained	14
X.	CONCLUSION.....	15

APPENDIX 1: Appealed Claims

APPENDIX 2: Exhibits

- Exhibit A — U.S. Patent No. 5,344,923
- Exhibit B — Hu *et al.*, (*PNAS* 89:9354-9358).
- Exhibit C — U.S. Patent No. 6,281,411
- Exhibit D — U.S. Patent No. 5,780,709
- Exhibit E — Barnett *et al.* (*Plant Phys.*, 41:1222, 1966)
- Exhibit F — Wyn Jones and Storey (*Physiology and Biochemistry of Drought Resistance in Plants*, Ch. 9, p. 171-204, Academia Press, Australia, 1981)
- Exhibit G — McCue and Hanson (*TIBTECH*, 8:358-362, 1990)
- Exhibit H — Van Rensberg *et al.* (*J. Plant. Physiology*, 141:1880194, 1993)
- Exhibit I — U.S. Patent No. 5,639,950
- Exhibit J — Rayapati *et al.* (*Plant Physiology*, 1989, 91:581-586)

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BRIEF ON APPEAL

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Appellants hereby submit an original and two copies of this Appeal Brief in response to the Final Office Action dated September 9, 2003. This Brief is filed pursuant to the Notice of Appeal mailed December 9, 2003. The date for filing the instant Brief is March 12, 2004, based on the receipt of the Notice of Appeal by the Patent and Trademark Office on December 12, 2003 and enclosed Petition for Extension of Time of One Month. The fees for filing this Appeal Brief are enclosed. If the check is inadvertently omitted, or should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to the enclosed materials, or should an overpayment be included herein, the Commissioner is authorized to deduct or credit said fees from or to Fulbright & Jaworski Deposit Account No. 50-1212/DEKM:184USD1.

Please date stamp and return the attached postcard as evidence of receipt.

PETITION FOR EXTENSION OF TIME

Pursuant to 37 C.F.R. § 1.136(a), Appellants petitions for an extension of time of one month to and including March 12, 2004, in which to file this Appeal Brief. Pursuant to 37 C.F.R. § 1.17, a check in the amount of \$440.00 is enclosed, which includes the process fee (\$110.00) for a one-month extension of time. If the check is inadvertently omitted, or should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to the enclosed materials, or should an overpayment be included herein, the Commissioner is authorized to deduct or credit said fees from or to Fulbright & Jaworski Deposit Account No. 50-1212/DEKM:184USD1.

I. REAL PARTIES IN INTEREST

The real party in interest is Monsanto Company, the parent company of assignee DeKalb Genetics Corp.

II. RELATED APPEALS AND INTERFERENCES

There are no interferences or appeals for related cases.

III. STATUS OF THE CLAIMS

Claims 1-58 were originally filed. Claims 59-96 were added and claims 1-58 were cancelled in the Preliminary Amendment mailed on December 7, 2000. In response to a Restriction Requirement, Appellants elected Group I, comprising claims 59-63 and 72-73. Claims 64-71 and 74-96 were subsequently withdrawn from consideration by the Examiner as drawn to non-elected subject matter.

Claims 59-63 and 72-73 were therefore pending and under consideration at the time of the Final Office Action. The final rejection of claims 59-63 and 72-73 is the subject of the instant appeal. A copy of the appealed claims is attached as **Appendix 1**.

IV. STATUS OF AMENDMENTS

An Amendment Under 37 C.F.R. §1.116 is being filed concurrently herewith clarifying that the seed of claim 73 includes the transgene referred to in claim 64. The status of the Amendment is thus unknown to Appellants. No other claim amendments have been made subsequent to the Final Office Action.

V. SUMMARY OF THE INVENTION

The invention relates to methods for the creation of transgenic monocot plants and seeds that display increased water stress resistance or tolerance. Specification at page 3, lines 20-24. More particularly, it concerns expression in monocots of an enzyme that catalyzes the synthesis of the osmoprotectant proline. Specification at page 3, lines 26-29, and page 4, line 8.

VI. ISSUES ON APPEAL

(A) Were claims 59-63 and 72-73 properly rejected under 35 U.S.C. § 112, first paragraph, as lacking an adequate written description in the specification?

(B) Were claims 59-63 and 72-73 properly rejected under 35 U.S.C. § 112, first paragraph, as lacking enablement in the specification?

(C) Were claims 61-63 and 73 properly rejected under 35 U.S.C. § 112, second paragraph, as being indefinite?

(D) Were claims 59-60 properly rejected under 35 U.S.C. § 102(e) as being anticipated by Verma *et al.* (U.S. Patent 5,639,950)?

(E) Were claims 59-63 and 72-73 properly rejected under 35 U.S.C. § 103(a), as being obvious over Verma *et al.* (U.S. Patent 5,639,950) in view of Rayapati *et al.* (*Plant Physiology*, 1989, vol. 91, pages 581-586) and Appellants allegedly admitted prior art?

VII. GROUPING OF THE CLAIMS

The claims stand or fall together for purposes of this appeal.

VIII. SUMMARY OF THE ARGUMENT

The Examiner has rejected the claims for lack of written description based on the alleged failure to disclose the genus of biosynthetic enzymes synthesizing proline. However, genes encoding proline were known to those of skill in the art at the time of filing (**Exhibit A** and **Exhibit B**). What was not known prior to the invention was that the genes could be expressed in monocots to yield water stress resistance. Given the art known status of proline-encoding genes, Appellants were in full possession of the genus. Reversal of the rejection is thus requested.

The Examiner has rejected the claims for lack of enablement based on an alleged failure to disclose the identification or isolation of a particular gene and/or plant comprising recombinant DNA encoding an enzyme involved in proline synthesis. However, Appellants' method for transformation of monocots combined with known examples of enzymes that catalyze synthesis of the osmoprotectant proline enable a person of ordinary skill in the art to produce transgenic monocots that express these enzymes. Appellants have demonstrated generally that expression of an osmoprotectant in monocots results in water stress tolerance (e.g., **Exhibit C** and **Exhibit D**). Further, numerous lines of evidence illustrate that proline was well known to be an osmoprotectant (**Exhibits E, F, G, and Exhibit H**). Appellants have therefore affirmatively set forth evidence of enablement, yet the Examiner has failed to present objective evidence supporting a reasonable doubt of enablement. Reversal of the rejection is thus requested.

The Examiner alleges that various terms used in the claims are indefinite. However, each of the terms used has a well known meaning clear to those of skill in the art in view of the specification and claim language. Reversal of the rejections is thus requested.

The Examiner alleges that claims 59-60 are anticipated by Verma *et al.* (U.S. Patent 5,639,950) under 35 U.S.C. § 102(e). However, Verma is not enabling for the transformation of

monocots. Additionally, Verma is effective only as of its June 1994 filing date for disclosure of “corn.” Neither “corn” nor synonymous terms “monocot” or “maize” appear in the priority application relied upon by Verma II. Reversal of the rejection is thus requested.

The Examiner has rejected claims 59-63 and 72-73 under 35 U.S.C. § 103(a), as being unpatentable over Verma *et al.* (U.S. Patent 5,639,950) in view of Rayapati *et al.* (*Plant Physiology*, 1989, vol. 91, pages 581-586). However, the reference fail to disclose the element of transgenic monocots generally, let alone monocots overexpressing proline or exhibiting water stress tolerance. The Examiner has further failed to show that one of skill in the art would have any reasonable expectation of success in expressing an enzyme catalyzing production of proline in monocots to yield water stress resistance. Thus, the Examiner has not made a *prima facie* case of obviousness and reversal of the rejection is therefore requested.

IX. ARGUMENT

A. The Written Description Rejection Was Improperly Maintained

The Examiner finally rejected claims 59-63 and 72-73 under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter that was not described in the specification in such a way as to convey to one of skill in the art that Appellants were in possession of the claimed subject matter. In particular, it was alleged that Appellants were not in possession of recombinant DNA molecules describing the genus of biosynthetic enzymes that catalyze the synthesis of the osmoprotectant proline.

In response, Appellants note that genes encoding enzymes that elevate the level of proline were known in the art at the time of filing. For example, Verma *et al.* (U.S. Patent 5,344,923, **Exhibit A**), which the Examiner cites in the prior art rejection, discloses the isolation of a mothbean cDNA clone encoding a bifunctional enzyme, delta1-pyrroline-5-carboxylate

synthetase, which is involved in the biosynthesis of proline in plants. Similarly, a soybean homologue of delta1-pyrroline-5-carboxylate synthetase was disclosed in 1992 by Hu *et al.* in *Proceedings National Academy of Science* (89:9354-9358; **Exhibit B**). Hu *et al.* also disclose that the enzyme catalyzes the first two steps in proline biosynthesis in plants. Verbruggen *et al.*, teach that pyrroline-5-carboxylate reductase encodes the last step of the proline biosynthetic pathway and describes the cloning of the corresponding gene from *Arabidopsis* (“Osmoregulation of a pyrroline-5-carboxylate reductase gene in *Arabidopsis thaliana*” *Plant Physiol.* Nov;103(3):771-81 (1993)). Additional examples of pyrroline-5-carboxylate reductases that have been described include the human (Dougherty *et al.* “Cloning human pyrroline-5-carboxylate reductase cDNA by complementation in *Saccharomyces cerevisiae*” *J. Biol. Chem.*, 267 (2), 871-875 (1992)) and yeast genes (Brandriss *et al.* “Proline biosynthesis in *Saccharomyces cerevisiae*: analysis of the PRO3 gene, which encodes, which encodes delta 1-pyrroline-5-carboxylate reductase” *J. Bacteriol.* 174 (15), 5176 (1992)).

As the sequences were known to those of skill in the art at the time of filing, Appellants cannot be said to lack written description for these sequences. A person of ordinary skill in the art would have known of useful gene sequences involved in the synthesis of proline at the time of Appellants’ invention. Appellants respectfully submit that the availability of such gene sequences as common knowledge obviates the rejection under 35 U.S.C. §112, first paragraph.

The evidence presented demonstrates full compliance with the written description requirement. Reversal of the rejection is thus respectfully requested.

B. The Enablement Rejection Was Improperly Maintained

The Examiner finally rejected claims 59-63 and 72-73 under 35 U.S.C. §112, first paragraph, as not being enabled by the specification. In particular, the Examiner takes issue with an alleged failure to disclose the identification or isolation of a particular gene and/or plant comprising recombinant DNA encoding an enzyme involved in proline synthesis.

In response, Appellants note that the claims are fully enabled by the specification. Specifically, as described in the specification, Appellants' method for transformation of monocots, combined with known examples of enzymes that synthesize the osmoprotectant proline, enables a person of ordinary skill in the art to produce transgenic monocots that express enzymes that synthesize proline.

The currently claimed invention forms a part of a larger invention comprising use of compatible osmoprotectants in monocots in order to achieve resistance to a reduction in water availability. For example, Appellants have illustrated this in the context of transgenic plants that express the osmoprotectant mannitol (see US Patent 5,780,709, **Exhibit D**) and glycine betaine producing enzymes (see U.S. Patent 6,281,411, **Exhibit C**). Proline, like mannitol and glycine betaine, is a known endogenous osmoprotectant and has long been identified as playing a role in plants under water deficit. Barnett *et al.* (*Plant Phys.*, 41:1222, 1966; **Exhibit E**) describes that under water deficit, significant increases in certain amino acid pools, such as proline, are observed. Wyn Jones and Storey (*Physiology and Biochemistry of Drought Resistance in Plants*, Chapter 9, p. 171-204, Academia Press, Australia, 1981; **Exhibit F**) notes that increased proline accumulation is observed in barley subjected to water or salt stress. McCue and Hanson (TIBTECH, 8:358-362, 1990; **Exhibit G**) specifically mention the amino acid proline as an osmoprotectant found in diverse organisms. Van Rensberg *et al.* (*J. Plant. Physiology*,

141:1880194, 1993; **Exhibit H**) discuss their observations of increased proline accumulation in drought-resistant tobacco cultivars, where a substantial amount of proline was found to accumulate in the drought-resistant cultivars compared to the drought-sensitive cultivars.

Appellants teaching that increased mannitol and glycine betaine impart water stress tolerance to transgenic monocot plants (see US Patent 5,780,709 **Exhibit D** and US Patent 6,281,411 **Exhibit C**, respectively) and success in using the mtlD gene to impart drought tolerance to a monocot are therefore indicative of success in overexpressing proline to obtain water stress tolerance. Together, the knowledge of proline accumulation in plants in response to drought stress, as well as the knowledge of sequences involved in the synthesis of proline demonstrate that the claims were enabled for expressing a proline biosynthesis gene and increased water stress tolerance. In fact, the Examiner's own prior art rejection states that expression of a delta1-pyrroline-5-carboxylate synthetase would inherently result in water stress tolerance. Given the transformation methods and expression vectors illustrated in Appellants' working examples to be fully enabling for monocot transformation and heterologous expression with osmoprotectant genes, it is respectfully submitted that the claims must be held enabled in view of the assertion. While legally flawed from an inherency rejection standpoint because the cited references are not enabling for transgene expression in maize and the insufficiency of Verma as of its effective date, the rejection affirmatively acknowledges the enablement of the claims given Appellants' enablement of transgene expression in monocots.

Reversal of the Section 112 rejection for lack of enablement is thus respectfully requested.

C. The Indefiniteness Rejections Were Not Properly Maintained

(1) Independent claim 61 and claims 62-63 dependent thereon stand rejected under 35 U.S.C. §112, second paragraph, as being indefinite for the use of the term “increased” in claim 61. It is stated that the term is relative, lacking comparative basis, and that the definition in the specification is not limiting.

Claim 61 reads as follows:

61. A fertile transgenic *Zea mays* plant comprising a recombinant DNA segment comprising a promoter operably linked to a first DNA segment encoding an enzyme which catalyzes the synthesis of the osmoprotectant proline, wherein the first DNA segment is expressed so that the level of the enzyme is *increased* in the transgenic *Zea mays* plant, and wherein the recombinant DNA segment is heritable.

(emphasis added)

As can be seen from the text of the claim, the meaning of “increased” is clear to one of skill in the art, particularly when taken in combination with the teaching in the specification. A plain reading of the claim indicates that the enzyme is increased relative to a *Zea mays* plant that lacks the recombinant DNA segment. No other logical reading can be made of the claim given the text of the claim. The Examiner has failed to point to any other reasonable meaning that could be given.

There is no indefiniteness in using a relative term that has a comparative basis. All that is required under the second paragraph of §112 is that one of skill in the art understand the metes and bounds of the claim when read in context and in view of the specification. Claim terms must be read together with the claim as a whole and in view of the understanding of those of skill in the art. Properly viewed, the claim is fully definite.

In light of the foregoing, reversal of the rejection under 35 U.S.C. § 112, second paragraph is respectfully requested.

(2) Claim 73 has been finally rejected under 35 U.S.C. §112, second paragraph, as being indefinite for the recitation of the term “transgenic seed.” The Examiner states that the seed may contain transgenes in addition to those used in the method of claim 68, thus it is not clear if the transgene referenced in claim 64 is included in the seed.

In response it is noted that an Amendment Under 37 CFR §1.116 is being submitted herewith that clarifies that the seed includes the transgene referred to in claim 64. It is believed that the rejection is moot in light of the Amendment.

D. The Anticipation Rejection Was Not Properly Maintained

The Examiner finally rejected claims 59-60 under 35 U.S.C. §102(e) as being anticipated by Verma *et al.* (U.S. Pat. No. 5,639,950, issued June 17, 1997, filed June 29, 1994 as a CIP of an application with an effective filing date of September 29, 1992 (hereinafter Verma II, **Exhibit I**)). The September 29, 1992 priority document of Verma II issued as U.S. Patent No. 5,344,923 (hereinafter Verma I, **Exhibit A**).

The rejection is insufficient on its face. Appellants noted in their response to the first Office Action that the current application claims priority to August 25, 1993 as a divisional application of U.S. Application Serial No. 08/599,714, filed January 19, 1996 (now U.S. Patent No. 6,281,411), which application was a continuation-in-part application of currently pending U.S. Application Serial No. 08/113,561, filed August 25, 1993. This grandparent application disclosed that transgenic monocot plants with drought resistance, including maize, can be prepared by expressing genes encoding a variety of osmotically active metabolites including proline.

The Verma II patent is effective only as of its June 1994 filing date for disclosure of “corn” and thus cannot anticipate the current claims. The Examiner nonetheless relies on Verma I based on the allegation that this disclosure is sufficient because there is allegedly no difference in the teachings with respect to transgenic plants. However, the shortcomings of Verma II do not supplement the shortcoming of Verma I. What is relevant is that Verma I is insufficient.

A full text search of the Verma I patent (U.S. Pat. No. 5,344,923) on the USPTO patent database reveals that this patent does *not include the terms “maize,” “Zea mays,” “corn” or “monocot”*. The Examiner’s rejection is therefore fundamentally without merit. It cannot be alleged that the patent anticipates transgenic monocots and maize expressing a particular transgene when it does *not even disclose monocot or maize plants* at all, let alone an enabling transformation method for such plants.

More particularly, Appellants’ claims are drawn to a transgenic monocot plant which is substantially tolerant or resistant to a reduction in water availability, where the transgenic plant comprises a transgene encoding an enzyme catalyzing the synthesis of proline. Verma I merely discloses the sequence of delta1-pyrroline-5-carboxylate synthetase, making the sequence available to those of ordinary skill in the art who might discover a use for it, *e.g.*, as Appellants have discovered a use in producing transgenic monocots expressing proline for drought resistance.

With regard to the Examiner’s statement that plants disclosed by Verma I and/or II would “inherently be substantially tolerant or resistant to a reduction of water availability” it is first noted that this statement directly contradicts the enablement rejection. The Examiner cannot have it both ways. In view of the comment by the Examiner admitting that a monocot transformed with the delta1-pyrroline-5-carboxylate synthetase gene would inherently have a

resistance to water availability, it is respectfully submitted that the Examiner has acknowledged the enablement of the claims.

It is further noted that the Examiner's conclusion regarding inherency is flawed. To form the basis of an inherency rejection, the missing item must necessarily flow from the disclosure. *Continental Can Co. USA v. Monsanto Co.*, 948 F.2d 1264, 1268, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991) ("To serve as an anticipation when the reference is silent about the asserted inherent characteristic, such gap in the reference may be filled with recourse to extrinsic evidence. Such evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill."). Here, the Examiner has failed to provide any objective basis to support the enablement of the Verma I or Verma II reference. For example, the Examiner has failed to show that Verma I and/or II disclose a method for transforming monocots and teach transformation vectors that could be used to achieve gene expression in monocots. Verma II does not even disclose monocots generally. The Examiner's unsupported allegations do not meet the relevant legal standards or the standards of the APA for maintaining a rejection.

The Examiner is not allowed to bootstrap deficient references to support an anticipation rejection through unsupported allegations of inherency. The law of inherency has been developed precisely so that the insufficiencies of a reference may not be glossed over by bare assertions of inherency. Given the failure of Verma I to disclose transgenic maize or monocots at all or a showing that Verma II is even prior art as of its priority date, there is no basis to allege that the claimed transgenic monocot plants expressing an enzyme catalyzing proline biosynthesis necessarily flow from the disclosure.

In view of the foregoing, neither Verma I nor Verma II constitute a 35 U.S.C. §102(e) bar to the present application. Reversal of the rejection is thus respectfully requested.

E. The Obviousness Rejection Was Not Properly Maintained

The Examiner rejects claims 59-63, and 72-73 under 35 U.S.C. §103(a) as being unpatentable over Verma II (described above) in view of Rayapati *et al.* (*Plant Physiology*, 1989, 91:581-586; **Exhibit J**) and in light of Appellant's allegedly admitted prior art. The teachings of Verma II are discussed above. Rayapati *et al.* is cited for its finding that delta1-pyrroline-5-carboxylate synthetase is located in the chloroplast of peas.

In response, Appellants note that all elements of the claims have not been shown in the art and thus the rejection is insufficient on its face. First, the Examiner has failed to show monocot plants in the prior art. Instead, the Examiner makes the conclusory statement that she "maintains that Verma *et al.* teach corn, wheat, barley, and rye monocot plants comprising a recombinant Δ^1 -pyrroline-5-carboxylate synthetase" that catalyzes proline synthesis. This assertion is unsupported and is baseless given that Verma does not even reference maize or monocot plants as of its effective prior date.

The Examiner has therefore failed to make a *prima facie* case of obviousness for the current claims, which are directed to transformed monocots having DNA which encodes an enzyme catalyzing the synthesis of the osmoprotectant proline. Verma II has not even been demonstrated by the Examiner to be properly used as prior art. Second, while Verma II claims a number of transgenic plant types, including maize, the reference does not disclose an actual transgenic monocot plant and thus does not cure the defect of Rayapati *et al.* The combined references do not teach fertile, transformed corn. The references have not even been shown to

motivate a person of ordinary skill in the art to attempt to transform corn, let alone provide a reasonable expectation of success in doing so.

Appellants respectfully submit that there is no support for a *prima facie* obviousness rejection of claims 59-63 and 72-73 over Verma II in light of Rayapati *et al.* Reconsideration and withdrawal of this Section 103 rejection is respectfully requested.

X. CONCLUSION

It is respectfully submitted, in light of the above, that none of the claims are properly rejected. Therefore, Appellants request that the Board reverse the pending grounds for rejection.

Respectfully submitted,



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Date: March 12, 2004

APPENDIX 1: APPEALED CLAIMS

59. A transformed monocot plant, which plant is substantially tolerant or resistant to a reduction in water availability, the cells of which comprise a recombinant DNA segment comprising a preselected DNA segment encoding an enzyme which catalyzes the synthesis of the osmoprotectant proline, wherein the enzyme is expressed in an amount effective to confer tolerance or resistance to the transformed plant to a reduction in water availability.
60. The transformed plant of claim 59 wherein the transformed plant has an improved osmotic potential when the total water potential of the transformed plant approaches zero.
61. A fertile transgenic *Zea mays* plant comprising a recombinant DNA segment comprising a promoter operably linked to a first DNA segment encoding an enzyme which catalyzes the synthesis of the osmoprotectant proline, wherein the first DNA segment is expressed so that the level of the enzyme is increased in the transgenic *Zea mays* plant, and wherein the recombinant DNA segment is heritable.
62. The fertile transgenic *Zea mays* plant of claim 61, wherein the recombinant DNA segment further comprises a second DNA segment encoding an amino terminal chloroplast transit peptide operably linked to the first DNA segment.
63. A seed produced by the transgenic plant of claim 61.

72. A transformed monocot plant regenerated from the transformed plant cells obtained by the method of claim 64.

73. A transgenic seed of the transformed plant of claim 72.

A bifunctional enzyme (Δ^1 -pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants

(osmoregulation/nitrogen assimilation/functional complementation/root nodules)

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Communicated by Jozef S. Schell, July 1, 1992

ABSTRACT Many plants synthesize and accumulate proline in response to osmotic stress. Despite the importance of this pathway, however, the exact metabolic route and enzymes involved in the synthesis of proline in plants have not been unequivocally identified. We report here the isolation of a mothbean (*Vigna aconitifolia*) cDNA clone encoding a bifunctional enzyme, Δ^1 -pyrroline-5-carboxylate synthetase (P5CS), with both γ -glutamyl kinase and glutamic- γ -semialdehyde dehydrogenase activities that catalyzes the first two steps in proline biosynthesis. The two enzymatic domains of P5CS correspond to the ProB and ProA proteins of *Escherichia coli* and contain a leucine zipper in each domain, which may facilitate inter- or intramolecular interaction of this protein. The *Vigna* P5CS enzyme activity is feedback regulated by proline but is less sensitive to end-product inhibition than is the *E. coli* γ -glutamyl kinase. The P5CS gene is expressed at high levels in *Vigna* leaves and is inducible in roots subjected to salt stress, suggesting that P5CS plays a key role in proline biosynthesis, leading to osmoregulation in plants.

Drought and high salinity are the most important environmental factors that cause osmotic stress and impact negatively on plant growth and crop productivity (1). To counter these stresses, many plants increase the osmotic potential of their cells by synthesizing and accumulating compatible osmolytes such as proline and glycine betaine (2-4). The role of proline biosynthesis in osmoregulation in bacteria is well established (5); however, it is not known whether the accumulation of proline in plant tissues confers some adaptive advantage to the plant under osmotic stress or whether it is a consequence of stress-induced changes in metabolism (6). A complete understanding of stress-induced changes in the proline biosynthetic pathway and the regulation of genes involved in proline metabolism may reveal how plants adapt to environmental stresses. This could open the way for increasing tolerance to drought and salinity stresses by producing transgenic plants capable of synthesizing elevated levels of proline.

The proline biosynthesis route in plants (7, 8) is thought to resemble the pathway in bacteria (9). In *Escherichia coli*, the reaction starts with the phosphorylation of glutamate by γ -glutamyl kinase (γ -GK; encoded by the *proB* gene), to form γ -glutamyl phosphate, which is reduced to glutamic- γ -semialdehyde (GSA) by GSA dehydrogenase (encoded by the *proA* gene). GSA spontaneously cyclizes to Δ^1 -pyrroline-5-carboxylate (P5C), which is reduced by P5C reductase (P5CR; encoded by the *proC* gene) to proline. The *E. coli* *proB*, *proA* (10), and *proC* (11) loci have been cloned and sequenced.

Whereas proline can be synthesized from either glutamate or ornithine in plants (7, 8) and animals (12-14), stable isotope

and radioisotope labeling experiments (15-17) indicate that glutamate, rather than ornithine, is the primary precursor for proline biosynthesis in osmotically stressed plant cells. With the exception of P5CR, which has been recently characterized (18-20),[‡] however, little is known about the other enzymes in this pathway. Although there is circumstantial evidence implicating a γ -GK in plants (7, 8), the existence of this activity has hitherto not been unequivocally demonstrated despite concerted efforts by several research groups over the past 25 years. Given the well-documented correlation between proline accumulation and adaptation to water deficits caused by drought and salinity stresses in plants, the complete characterization of the enzymes in the proline biosynthetic pathway and their biochemical and genetic regulation is vitally important.

We have recently isolated cDNA clones for soybean P5CR by direct complementation of an *E. coli* *proC* proline auxotroph with a soybean nodule cDNA expression library (20). The rationale of using a nodule library was based on the finding that high levels of proline are synthesized in soybean root nodules (21, 22). Using this complementation strategy (23), we have isolated from a mothbean (*Vigna aconitifolia*) nodule cDNA expression library cDNA clones coding for a bifunctional enzyme, P5C synthetase (P5CS), which exhibited both γ -GK and GSA dehydrogenase activities. The *Vigna* P5CS enzymatic activity was found to be feedback inhibited by proline. The level of P5CS transcript was shown to be enhanced in roots treated with salt, indicating that the expression of this gene is osmoregulated.[§]

MATERIALS AND METHODS

Bacterial Growth Conditions. *E. coli* mutant cells were grown at 37°C in LB medium, or in minimal A medium (23) supplemented with 0.2 mM required amino acids and 0.05 mM thiamine; ampicillin was added at 100 mg/liter. The osmotic strength of the medium was increased by the addition of NaCl as indicated.

Plasmids and Bacterial Strains. Plasmids carrying wild-type *proB* (pDU1) and mutant *proB74* (pDU101) alleles were obtained from Abhaya Dandekar (University of California, Davis). *E. coli* strains X340 (*proB28*, *metB1*, *relA1*, λ^- , *spoT1*), W4032 (*proA3*, *metB1*, *relA1*, *lac-3*, *tsx-76*), CSH26 [see ref. 24; *ara*, Δ (*lac pro*), *thi*], and X342 (*proC29*, λ^- ,

Abbreviations: γ -GK, γ -glutamyl kinase; GSA, glutamic- γ -semialdehyde; P5C, Δ^1 -pyrroline-5-carboxylate; P5CR, P5C reductase; P5CS, P5C synthetase.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M92276).

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relA1, *sptT1*, *metB1*) were kindly provided by Barbara Bachmann (*E. coli* Genetics Stock Center, Yale University).

Construction of cDNA Expression Library. RNA was prepared from 21-day-old mothbean (*Vigna aconitifolia* L.) nodules by the method of Verwoerd *et al.* (25), and poly(A)⁺ RNA was isolated by chromatography on an oligo(dT)-cellulose column. Double-stranded cDNA was synthesized, ligated to *Bst*XI linkers, and size-fractionated on an agarose gel. Molecules >500 base pairs were electroeluted from the gel, cloned into the *Bst*XI-linearized pCDNAII plasmid vector (Invitrogen, San Diego) and *E. coli* DH1 α F' cells were transformed. cDNA molecules inserted within the polylinkers of pCDNAII in the correct orientation and reading frame can be expressed under the control of the *lacZ* promoter.

Functional Complementation of *E. coli* Auxotrophs. *E. coli* proline auxotrophs were transformed by electroporation using Electro Cell Manipulator 600 apparatus (BTX, San Diego) according to the manufacturer's instructions. Briefly, cells were grown to late logarithmic phase in LB broth, pelleted by centrifugation, washed twice in ice-cold 10% (vol/vol) glycerol, resuspended in cold 10% glycerol, and stored in 200- μ l aliquots at -80°C. For electroporation, 20 ng of the pooled library DNA purified by double CsCl centrifugation was added to 200 μ l of competent cells in a 2-mm cuvette and subjected to a 5-msec pulse at 2.5 kV/cm. The cells were immediately added to 10 ml of LB broth and incubated at 37°C with shaking for 1 h. The cells were then pelleted by centrifugation, washed with minimal A medium, and plated onto minimal A plates containing ampicillin (100 μ g/ml), isopropyl β -D-thiogalactopyranoside (1 mM), and the amino acid supplements other than proline required by the respective mutants. To determine the transformation efficiencies, aliquots of electroporated cells were plated onto the same media supplemented with proline (1 mM). Plates were incubated at 37°C for 2 days. Plasmid DNA, prepared from single colonies grown in minimal medium containing ampicillin, was used to retransform the mutants to confirm the complementation (see ref. 23).

DNA Sequencing and Analysis. DNA sequencing was performed on CsCl-purified, double-stranded plasmid DNA by the dideoxynucleotide chain-termination method using Sequenase II (United States Biochemicals) according to the manufacturer's instructions. Deletions of the plasmid template were generated by using Exonuclease III and mung bean nuclease. In addition, some sequencing reactions were performed with oligonucleotide primers synthesized on the basis of previously derived cDNA sequences. Sequence comparisons and identification of various motifs were done with Genetics Computer Group programs (Madison, WI).

P5CS Enzyme Assay and Determination of End-Product Feedback Inhibition. *E. coli* CSH26 cells and CSH26 harboring different plasmids were grown aerobically at 37°C for \approx 16 h. The cells were harvested by centrifugation at 4000 \times g for 10 min, and the cell paste was suspended in 4 ml of buffer A (50 mM Tris-HCl/1 mM dithiothreitol) at 4°C. After sonication (Benson Sonifier, type 450) at 50 W, the slurry was centrifuged at 4000 \times g for 10 min and the supernatant was fractionated by ammonium sulfate precipitation. For *E. coli* CSH26 containing pVAB2, the fraction precipitating between 0% and 30% saturation of ammonium sulfate was used for enzyme assay; while for *E. coli* CSH26 and CSH26 harboring the *proB74* or wild-type *proB* alleles, a fraction of 40–60% saturation was used. Ammonium sulfate precipitated fraction was dissolved in buffer A and dialyzed against the same buffer. γ -GK activity was assayed by the method described by Hayzer and Leisinger (26). The amount of γ -glutamylhydroxamate made from L-glutamate, NH₂OH, and ATP was determined from the A₅₃₆ after a 30-min incubation at 37°C. To determine whether proline biosynthesis is subject to end-product inhibition in plants, the γ -GK activities of the

different ammonium sulfate fractions described above were measured as a function of proline concentration (0.01–100 mM) in the assay mixture.

Northern Blot Analysis. Poly(A)⁺ RNA from mothbean nodules, leaves, and roots, as well as roots from plants watered with 200 mM NaCl, were electrophoresed in a 1.2% agarose/2.2 M formaldehyde gel and transferred by vacuum blotting to a GeneScreen membrane (DuPont/NEN). The pVAB2 cDNA insert was labeled with [³²P]dCTP to a specific activity of 8 \times 10⁸ dpm/ μ g using a random-primer labeling system (Amersham). Hybridization was performed as described (20), and filters were exposed to x-ray film at -70°C.

RESULTS AND DISCUSSION

Isolation of cDNA Clones Encoding *Vigna* P5CS. We have previously obtained a cDNA clone for soybean P5CS by direct complementation of an *E. coli* *proC* proline auxotroph with a soybean nodule cDNA expression library (20). To clone other plant enzymes of the proline biosynthetic pathway, we similarly transformed *E. coli* *proB*, *proA*, and *proBA* auxotrophic mutants by electroporation with a *V. aconitifolia* nodule cDNA expression library. A transformation efficiency of 10⁹–10¹⁰ transformants per μ g of DNA was routinely obtained, which is \approx 2 orders of magnitude higher than that obtained by classical transformation methods (23); thus, electroporation significantly enhances the recovery of complemented mutants. One group of clones was isolated by selecting for growth of transformants on minimal medium without proline. We have determined that these clones encode a bifunctional enzyme, P5CS, which has both γ -GK and GSA dehydrogenase activities. This determination is based on the following evidence: the P5CS clones efficiently complemented *E. coli* *proB*, *proA*, and *proBA*, but not *proC*, mutants; the predicted amino acid sequence of the enzyme revealed the presence of two domains with significant homology to the *E. coli* ProB and ProA proteins; and the recombinant P5CS protein showed GSA-dependent γ -GK enzyme activity.

P5C can also be synthesized from ornithine via ornithine aminotransferase in plants, but the *E. coli* CSH26 cells are unable to grow on ornithine. These cells electroporated with library DNA were selected for proline prototrophy on minimal medium supplemented with ornithine (1 mM). With this approach, we obtained complementing clones encoding ornithine aminotransferase (unpublished data). These data demonstrate that proline is synthesized in plants from both glutamate and ornithine. The contribution of the respective pathway to proline production remains to be determined.

***Vigna* P5CS Contains Domains Homologous to *E. coli* ProB and ProA Proteins.** The complete nucleotide sequence of a P5CS cDNA clone, pVAB2, is shown in Fig. 1. The 2417-base-pair sequence contains a single major open reading frame encoding a polypeptide of 73.2 kDa, assuming that translation is initiated at the first ATG codon from the 5' end of the coding strand. Sequence comparison with the *E. coli* ProB and ProA proteins (Fig. 2) indicated that *V. aconitifolia* P5CS polypeptide has two distinct domains. The amino-terminal domain of the P5CS protein showed 33.3% identity and 55.3% overall similarity to the *E. coli* ProB protein. A domain with 35.7% identity and 57.9% similarity to the ProA protein is located at the carboxyl end. An unexpectedly high level (42.4%) of sequence similarity was found between the *E. coli* ProB and ProA proteins, suggesting that the *proB* and *proA* genes may have arisen by duplication of an ancestral gene; the encoded proteins may have later acquired domains conferring the respective kinase and reductase activities of the present-day enzymes. The corresponding plant genes have evidently fused to create the bifunctional enzyme identified in *V. aconitifolia*. A similar event may have occurred

A

VPBA 12	MKDVKRVIIKVGTA VVTREGRVAGRLGALCEQIKQLNSLCYDIILVSS	61
EPB 1	MSDSQTLVVKLGTSVL TGGSRRLNRHIVELVRQCAQLBAAGHRIVTS	50
VPBA 62	GPVIGIGRQRLFRKLI NSFPADLQKPOLELDGKACAAVGNLSMALYDTL	111
EPB 51	GAIAAGREHLGYPELP... ATIASKQLL... AAVGQSRLIQLMEQL	90
VPBA 112	FTQLDVTSAQLLVTDN FFRKDFRKQLTETVKSLLAKVIPVFNENDAVS	161
EPB 91	FSIYGIHVGOHLLTRF MEDRERFLNARDTLRALDNNIVPVINENDAVA	140
VPBA 162	TPKAPYEDSSGIFWMDN SLLALLLEKADLLVLLSDVEGIV. SGPPSDP	210
EPB 141	TAAIKVG... DNDNLSALAAIAGADKLLLTQKGLYTADPRSNP	183
VPBA 211	HSKLI. YTYNKEKHQWE ITFGKSRVRCGGM TAKVKAHVAAAGIPVVI	259
EPB 184	QAEIKLDVYGD DALRAIARD SVSGLTGGHSTKLAADVACRAGIDTII	233
VPBA 260	TSGFAPENINVLOQGRIG TLFHKDAHEWAQVKEVDAREMAVAAGNVREG	309
EPB 234	AAGSKPGVIGDVGESVGT LPHAQTPLENRKRWIFGAPPAGEITVDEG	283
VPBA 310	SRRYLQRKGNKILLKIA DALEAN... EKIRIENEADVTAAGAGYEKSL	356
EPB 284	ATAAILERGSLLPKGIK SVTGNFSRGEVIRICNLEGRDIAGVSRNS.	332
VPBA 357	VARLALKPGKIASLANM RRIANMEDPIGRVLRTELDGILILEKTSPL	406
EPB 333 DALPRIAGHSQEI DAILCYTEYGPV	357
VPBA 407	GVLLIVFESRPDALV 421	
EPB 358	AV.....HRDDMI 365	

B

VPBA 298	EMAVAAGNVREGSRRYL QRKGNKILLKIA DALEANEKIRIENEADVTA 347
EPA 4	QMGIAAKQASYKLAGL SSSREKRVLEKIADELEAQSEIILNANAQOVADA 53
VPBA 348	QEAGYEKSLVARLALK PGKIASLANM RRIANMEDPIGRVLRTELDG 397
EPA 54	RANGLSEAMLDRIALT PAKLGIADDRVQVCNLADPVGVGGVLDGSL 103
VPBA 398	ILEKTSPLGVLLIVF ESFPDALVQIASLAIRSGNLLKGGKEAKRSNA 447
EPA 104	RLERRRVP LGVIGVYEARPNVTVDVASLCLKTGNNAVILRGCKETCRNA 153
VPBA 448	ILHKVITAEIPD. NVGGKLI GLV. TSREDPPELLKDDVIDLVIPRGSN 494
EPA 154	ATVAVIQDALKSCGLP AGAVQIDNPDRALVSEMLRMDKYIDMLIPRGA 203
VPBA 495	KLVSQIKSSKTIPIVL GADGICHVTVKDSANVEMAKRIVLDAKVDPYAPAC 544
EPA 204	GLHKLREQSTIPVIT GGGVCHIVYDESVEIAELKVIVNAKTQRPSTC 253
VPBA 545	NAMETLLIHKLT EKGWLEKIIIDLRTEGVIDFGGPFVASSLLNIP. 589
EPA 254	WTETLLVWKN. IADSFPLSKQMAESGVTLHADAAALQQLQAGPAKV 302
VPBA 590	..QAHSPHNEYSSLA CTAEIVDDVYAAIDHINLYGSAHTDSIVAEDEVA 637
EPA 303	AVKAEYDDEFLSLDL NVKIVSOLDDAIAHIREHGTOHSDAILT. 346
VPBA 638	NVFLRQVDSAAVFNH NASTRFS DGAPE 664
EPA 347RDMRQAQRFVNA STRFTDGGQGF 369

Fig. 2. Comparisons of the *V. aconitifolia* P5CS amino acid sequence (VPBA) with the *E. coli* γ -GK (EPB) (A) and GSA dehydrogenase (EPA) (B) sequences (10). Identical amino acids are indicated by a vertical line; similar amino acids are indicated by colons and periods. Conserved aspartic acid residues implicated in proline feedback inhibition are boxed, as are the leucine zipper sequences and the potential phosphorylation sites.

than the mutated γ -GK enzyme. The mutation responsible for the resistance to end-product inhibition of γ -GK in the *proB74* allele involves a nucleotide substitution of an A for a G, resulting in a change from aspartic acid to an asparagine residue (29, 33). We note that this aspartate residue is conserved in the *Vigna* P5CS enzyme, raising the possibility that changing this residue to asparagine by site-directed mutagenesis may result in further diminution of feedback

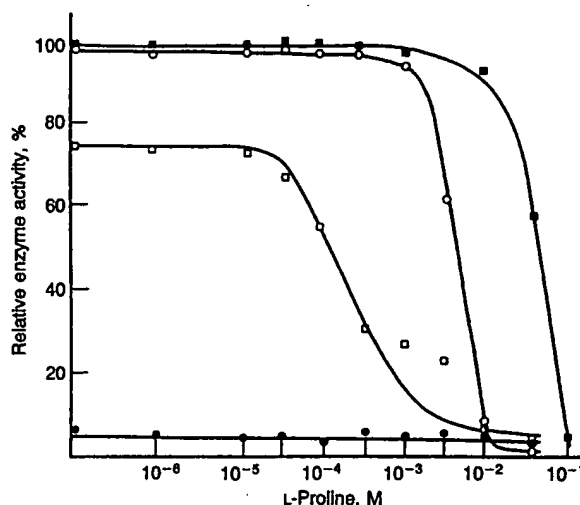


Fig. 3. Feedback inhibition of P5CS by proline. Ammonium sulfate-fractionated extracts (26, 29) from *E. coli* CSH26 (●), *E. coli* CSH26 harboring pVAB2 (○), the *proB74* allele (■), and the wild-type *proB* allele (□) were assayed for γ -GK activity by the method of Hayzer and Leisinger (26).

inhibition of the plant enzyme. In spite of the conserved aspartic acid residue implicated in feedback control, the *Vigna* enzyme is less sensitive to inhibition by proline than the *E. coli* enzyme, suggesting that other regions of the P5CS enzyme may also be important for feedback regulation.

By analogy with the proline biosynthetic pathway in *E. coli* and given the sensitivity of the mothbean enzyme to end-product inhibition, it seems likely that regulation of the pathway in plants is exerted primarily at the P5CS step. The synthesis of P5C in animals, however, is not inhibited by L-proline but is sensitive to ornithine (12–14). In this regard, the plant enzyme is more similar to that in prokaryotes. However, P5C can be made from ornithine in *Vigna* (unpublished data), and therefore the relative contribution of glutamate or ornithine to proline may depend on the availability of nitrogen.

Proline Biosynthesis Genes Are Osmoregulated. We have previously demonstrated that expression of the soybean P5CR gene is enhanced at the RNA level in nodules and in osmotically stressed soybean roots (20), a phenomenon recently confirmed in salt-stressed *Arabidopsis thaliana* leaves.⁵ To investigate the expression levels of the *Vigna* P5CS gene in different tissues and under conditions of osmotic stress, RNA from nodules, leaves, and roots, as well as roots from plants watered with 200 mM NaCl, was probed on a Northern blot with the pVAB2 cDNA. As shown in Fig. 4, the P5CS transcript in *Vigna* leaves is more abundant than in roots and nodules. The level of P5CS transcripts in roots is enhanced by treatment of the plant with 200 mM NaCl. An increase in the level of transcript of P5CS and P5CR during osmotic stress may facilitate proline production from glutamate. However, the P5CR reaction in plants is not normally rate-limiting (34, 35). The elevated level of P5CS may control the flux of glutamate to P5C. Thus, the proline synthesis pathway is not only feedback regulated but also controlled transcriptionally. The latter is also influenced by the level of nitrogen in the cell.

The elevated rate of proline biosynthesis in nodules has been suggested to stimulate ureide synthesis in tropical legumes and to help transfer redox potential from the nodule cytoplasm to the bacteroids (21, 22). Proline may also act as a carbon and nitrogen source for the bacteroids. An addi-

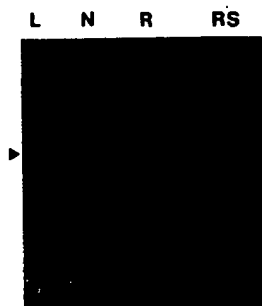


FIG. 4. Northern blot analysis of poly(A)⁺ RNA (5 μ g each) from leaf (lane L), nodule (lane N), root (lane R), and root treated with 200 mM NaCl (lane RS), probed with the ³²P-labeled pVAB2 cDNA insert. Arrowhead indicates the 2.6-kilobase P5CS transcript.

tional probable role of proline in nodules may be its involvement in osmoregulation (20). The osmoticum in infected nodule cells is known to be 4- to 5-fold higher than in root cells (36). Our demonstration that both P5CS and P5CR (20) genes are induced by salt stress is consistent with the hypothesis that accumulation of proline in nodules may represent an osmoregulatory adaptation to increased concentration of solutes. Recently, a clone encoding γ -GK has been identified from a tomato cDNA library and is also suggested to contain GSA dehydrogenase activity.[†] The availability of a cloned plant gene encoding the rate-limiting enzyme in proline biosynthesis will allow understanding of the role of proline in osmoregulation. This opens the way for genetically manipulating this pathway in transgenic plants, with the potential for increasing proline production, which may confer tolerance to drought and salinity stresses in crop plants.

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Amino Acid and Protein Metabolism in Bermuda Grass During Water Stress^{1,2}

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Summary. The ability of Arizona Common and Coastal Bermuda grass [*Cynodon dactylon* (L.) Pers.] to synthesize amino acids and proteins during water stress was investigated. Amino acids were continually synthesized during the water stress treatments, but protein synthesis was inhibited and protein levels decreased.

Water stress induced a 10- to 100-fold accumulation of free proline in shoots and a 2- to 6-fold accumulation of free asparagine, both of which are characteristic responses of water-stressed plants. Valine levels increased, and glutamic acid and alanine levels decreased.

¹⁴C labeling experiments showed that free proline turns over more slowly than any other free amino acid during water stress. This proline is readily synthesized and accumulated from glutamic acid. It is suggested that during water stress free proline functions as a storage compound.

No significant differences were found in the amino acid and protein metabolism of the 2 varieties of Bermuda grass.

In the study of biochemical changes in plants under water stress conditions, increasing attention has been paid to changes in nitrogen compounds. Proteolysis and interruption of protein synthesis are generally found to be results of water stress (6,11,20), although both increases and decreases of protein have been found to follow each other (3). Radioisotopes have been used to show the effects of water stress on RNA synthesis and degradation (4). The study presented here reports the effects of water stress on levels and turnover of both free and protein-bound amino acids as shown by ¹⁴C labeling.

Water stress induces a characteristic change in the levels of free amino acids, especially a great increase in free proline (3,6,12) and amides (3,9). The accumulation of amides is thought to be the result of incorporation of free ammonia released by deamination of amino acids, which were in turn released by proteolysis induced by water stress (9). Few attempts have been made to explain the accumulation of free proline. The origin and function of this proline is considered in this paper.

Two varieties of Bermuda grass have been used in the present study. These varieties differ some-

what in their general response to water stress, and it was desired to see if under water stress conditions differences also exist in their nitrogen metabolism. An extensive study by Ratnam (13) showed these differences in drought response of Arizona Common and Coastal varieties: Water content and cuticular transpiration are higher in Common Bermuda. Common Bermuda leaves develop a lower (more negative) water potential in a given time without water than do Coastal Bermuda leaves. Leaf damage is generally greater and appears sooner in Common than in Coastal leaves. In general, Ratnam's experimental results tend to support the conclusion that leaves of Coastal are slightly superior to Common in drought avoidance.

Materials and Methods

Plant Material. Clonal material of Arizona Common and Coastal Bermuda grass [*Cynodon dactylon* (L.) Pers.] was propagated in a 2:1 mixture of sandy loam and sand in 7 inch clay pots. Plants were grown in the greenhouse and were fertilized periodically with commercial fertilizer. The grasses were transplanted into new soil-sand mixture when growth ceased to be vigorous. Tops were cut off periodically. Experiments were conducted in fall or winter, when growth was slow and there was no flowering.

Water Potential Measurement. Water potential was measured with the thermocouple psychrometer device described by Boyer (2).

Labeling of Plants with ¹⁴CO₂. Labeling ex-

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periments were conducted in an isotope hood. Two adjacent rows of plants in clay pots were illuminated from opposite sides with 150-w reflector spot lamps so that shading was minimized. Light was filtered through 9 cm of 0.5 % copper sulfate solution in glass tanks cooled by tap water flowing through copper coils. Light intensity was 1000 ft-c at the leaf surface. Plants were left in place throughout the labeling and sampling periods. Daylength was 8 hours.

For incubation with $^{14}\text{CO}_2$, a 15.8 liter bell jar was placed over a plant. Bell jar and plant rested on a glass plate, to which the bell jar was sealed with silicone grease. A 10 ml beaker containing 200 μC of $\text{NaH}^{14}\text{CO}_3$ (specific activity 25 μC μmole^{-1}) was suspended on a wire inside the top of the bell jar. The bell jar top was sealed with polyvinyl chloride film. To generate $^{14}\text{CO}_2$, 0.1 ml of 20 % lactic acid was injected through the film into the beaker; the film was immediately sealed with Scotch tape. After one-half hour, 0.5 ml of concentrated NaOH was injected into the beaker. The bell jar was removed five minutes later. The maximum concentration of CO_2 generated was 0.0012 %, which is small compared to the normal concentration of CO_2 in air. No artifacts due to high CO_2 concentration were likely to have been induced.

Extraction of Free Amino Acids. Plant tissue was killed by boiling it for 3 minutes in 80 % (v/v) ethanol. Tissue and ethanol were stored at -20° . The ethanol was subsequently decanted and saved, and the tissue was ground with mortar and pestle with acid-washed sand and fresh 80 % ethanol. The homogenized sample was refluxed 15 minutes on a steam bath. The sample was centrifuged 15 minutes at 27,000 g. The supernatant fraction was added to the original ethanol in which the tissue was killed. The pellet was refluxed again in 40 % ethanol. This procedure of refluxing and centrifuging was done 4 times in all, once with 80 % ethanol, twice with 40 % ethanol, and once with water. All supernatant fractions were pooled. Four extractions yielded 94 % of the free amino nitrogen obtained in 6 extractions (80 % ethanol, twice in 40 % ethanol, 3 times in water). Pooled extracts were further purified by evaporation almost to dryness at 45° under reduced pressure, taking up the residue in 2 ml of 0.1 N HCl, and centrifuging the suspension 10 minutes at 1° at 27,000 g. This procedure was repeated once or twice; the pellet was discarded each time. Extracts were then purified by the cation exchange method of Wang (18). Recovery of free amino nitrogen in this method was 91 %. This solution was reduced to dryness and the residue was taken up in a small amount of 0.1 N HCl.

Analysis of Amino Acids. Amino acids were measured on an automatic amino acid analyzer using the J-column technique and buffer sequence of Picz and Morris (10). The analyzer was cali-

brated with standard mixtures of amino acids. At the column temperature of 60° , glutamine is cyclized to pyrrolidone carboxylic acid, which does not react with ninhydrin. Consequently sample glutamine was not measured. Radioactivity of the analyzer stream was monitored continuously with a Packard 317 scintillation detector and 320E pulse height analyzer. One channel of the amino acid analyzer recorder was used to record radioactivity.

Extraction of Soluble Protein. One grass shoot (up to 15 cm high and 0.5 g dry weight) was cut into 1.5 cm segments and ground with 2 ml water and acid-washed sand with mortar and pestle at 1° . The homogenate was centrifuged at 27,000 g at 1° for 10 minutes. The supernatant fraction was saved. The pellet was ground again with water and sand at 1° and recentrifuged. The water soluble protein in the combined supernatant fraction was precipitated by adding an equal volume of 20 % trichloroacetic acid (TCA) and allowing to settle at least 10 minutes. The protein was centrifuged at 27,000 g for 10 minutes, the pellet was resuspended in 10 % TCA and recentrifuged; then the supernatant was discarded. The pellet was decolorized by twice incubating at one-half hour at 37° with 2 ml of a 2:2:1 (v/v/v) mixture of ethanol, ether, and chloroform, and centrifuging each time. The protein precipitate was dissolved overnight in 1 ml of 1 N NaOH.

Protein Measurement. Protein was measured both by the method of Lowry et al. (8) and by summing the amino acids in protein hydrolysates as measured on the analyzer.

Hydrolysis of Protein. Protein solutions were made to 6 N HCl in 2-piece hydrolysis tubes. After evacuation of air, the solutions were hydrolyzed at 110° for 20 hours. The small amount of humic acid formed was removed by filtration. Hydrolysates were dried on a flash evaporator at 45° . Water was added to the residue and the sample was redried repeatedly to remove excess HCl. The hydrolysate was dissolved in 1 ml 3 M citric acid for analysis on the amino acid analyzer.

Results

In addition to the water soluble protein amino acids, several other amino acids were detected by column chromatographic analysis of ethanolic extracts of Bermuda grass tops. On the basis of elution time and comparative color yield (blue/yellow absorption) the non-protein amino acids α -aminobutyric acid, β -alanine, and pipercolic acid were tentatively identified. The last 2 were present in minute amounts. Extracts of large samples of whole tops revealed minute quantities of still more unidentified ninhydrin-positive compounds. This is illustrated in figure 1 where many small unidentified peaks are shown. Two such compounds were present, however, in amounts large enough to measure. One, labeled N throughout

1224

PLANT PHYSIOLOGY

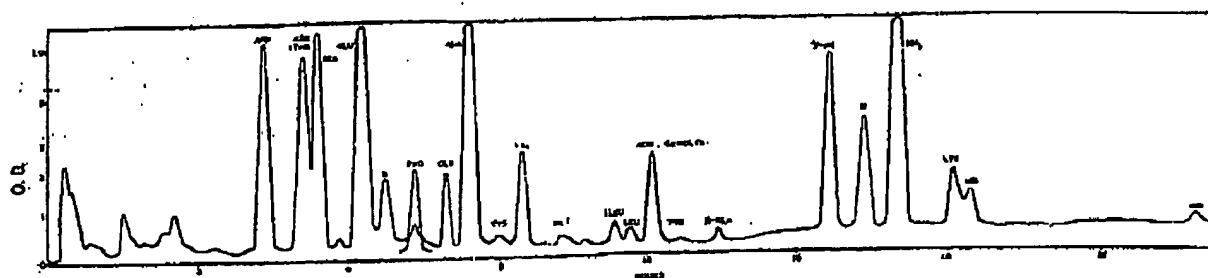


FIG. 1. Chromatogram trace of free amino acids of Bermuda grass produced by the automatic amino acid analyzer. Norleucine (4 μ moles) was added to the sample of fresh tops as an internal standard.

this study, was eluted between glutamic acid and proline. It emerged from the column about 15 minutes after standard citrulline, and disappeared upon hydrolysis with acid.

The other unknown, labeled U, was eluted between α -amino-butyric acid and ammonia. It emerged from the column at the same time as standard ethanolamine and δ -allo-hydroxylysine. It was stable to acid hydrolysis. In an attempt to identify this unknown, 2.4 ml fractions were collected during an amino acid analysis of an extract of 136 g fresh Coastal Bermuda grass tops. The solutions in the 3 tubes containing the unknown U were pooled and deionized on a 0.9×10 cm column of Dowex 50×8 , NH_4 form (7). The ammonia solution of the unknown was dried at 40° under

reduced pressure. The residue was dissolved in 1 ml 0.1 N HCl. Two 1-dimensional chromatograms were run, using 50 μ l of unknown, plus standards, on Whatman No. 1 chromatography paper. Solvent systems were 71% phenol, and *n*-butanol-propionic acid-water [45.3:22.5:32.2, v/v/v (1)]. The unknown chromatographed the same distance as ethanolamine in both systems (phenol-water: unknown R_F .70, ethanolamine R_F .68; *n*-butanol-propionic acid-water: unknown R_F .55, ethanolamine R_F .52). The unknown U was therefore tentatively identified as ethanolamine.

An experiment was conducted to determine the effects of water stress on the composition and turnover of both free and protein-bound amino acids. Duplicate sets of both Arizona Common

Table I. Effect of Water Stress on Fresh Weight, Dry Weight, Total Free Amino Acids, and Water-soluble Protein in Bermuda Grass Shoots

Treatment	Water potential range, bars	Fr. wt, mg*	Dry wt, mg*
Common Control	-4.1 to -7.9	228.6 \pm 102.9	46.3 \pm 23.2
Moderate stress	-10 to < -37	174.4 \pm 69.2	67.6 \pm 25.9
Severe stress	< -37	84.6 \pm 36.1	61.3 \pm 28.6
Coastal Control	-4.1 to -4.7	341.3 \pm 116.3	69.4 \pm 24.2
Moderate stress	-18 to < -37	179.6 \pm 48.6	52.4 \pm 13.9
Severe stress	-33 to < -37	128.9 \pm 61.7	82.6 \pm 29.7

Total free amino acids, μ moles per shoot*	Water-soluble protein: μ moles hydrolyzed amino acids per shoot*	mg/shoot	Total amino N, μ moles per shoot
8.72 \pm 3.49	18.9** \pm 3.5	3.06	27.6
13.9 \pm 5.71	12.6 \pm 4.7	2.11	26.5
16.7 \pm 4.3	9.31 \pm 2.41	0.95	25.0
9.26 \pm 2.68	23.7 \pm 11.4	3.24	33.0
20.4 \pm 7.8	13.5 \pm 5.3	2.07	33.9
28.0 \pm 9.8	9.31 \pm 4.13	1.07	37.3

* Average and standard deviation of 5 determinations made in a 77-hour period.

** Four determinations only.

BARNETT AND NAYLOR—AMINO ACID AND PROTEIN METABOLISM

1225

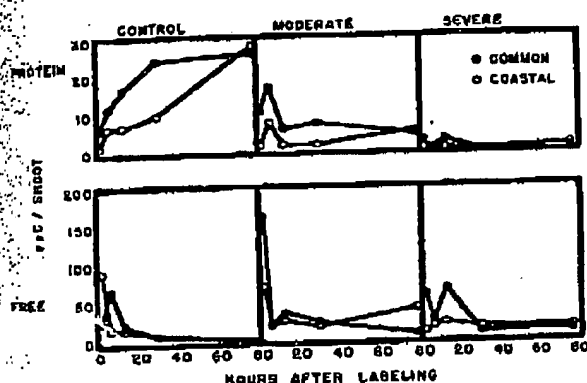


FIG. 2. Time course of change in radioactivity of the free amino acid fraction and soluble protein fraction in Bermuda grass with increasing water stress.

Table II. Changes in Amounts of Free Amino Acids in Bermuda Grass Shoots with Increasing Water Stress

Increasing water stress

Amino acid	Control	μ moles/gram dry weight**		Severe stress
		Moderate stress		
Common				
Aspartic acid	11.8 \pm 8.9	4.5 \pm 1.8		8.4 \pm 3.5
Asparagine; threonine	24.6 \pm 4.1	29.8 \pm 13.7		64.2 \pm 17.1
Serine	9.9 \pm 2.3	8.3 \pm 2.7		11.0 \pm 4.5
Glutamic acid	28.7 \pm 9.2	10.5 \pm 4.8		4.7 \pm 1.9
N		0.8 \pm 0.3
Proline	< 2.7	30.5 \pm 23.9		69.3 \pm 33.0
Glycine	1.8 \pm 1.3	1.7 \pm 1.1		1.2 \pm 0.7
Alanine	31.9 \pm 12.3	15.2 \pm 3.8		11.6 \pm 4.2
1/2-Cystine		0.6** \pm 0.1
Valine	2.1 \pm 0.7	3.5 \pm 1.6		7.0 \pm 2.1
Isoleucine	...	0.9** \pm 0.4		1.2 \pm 0.4
γ -Aminobutyric acid	3.2 \pm 2.1	7.0 \pm 4.1		4.3 \pm 1.4
U	...	0.8 \pm 0.2		1.5 \pm 0.9
Ammonia	94.3 \pm 36.6	78.0 \pm 54.0		55.4 \pm 26.4
Lysine	0.5 \pm 0.4	0.7 \pm 0.1		1.0 \pm 0.4
Histidine	...	0.5 \pm 0.1		1.4 \pm 0.5
Arginine	...	0.8 \pm 0.3		2.5 \pm 0.1
Totals	211.5	192.9		246.5
Coastal				
Aspartic acid	7.0 \pm 5.9	9.0 \pm 4.2		9.7 \pm 1.7
Asparagine; threonine	9.4 \pm 3.3	60.1 \pm 21.5		62.5 \pm 29.9
Serine	7.9 \pm 1.3	18.5 \pm 7.9		13.4 \pm 6.3
Glutamic acid	22.3 \pm 7.7	17.7 \pm 6.5		5.4 \pm 2.3
N	0.9 \pm 0.1	1.3 \pm 0.3		0.7 \pm 0.3
Proline	< 1.1	138.0 \pm 64.0		126.0 \pm 34.0
Glycine	0.8 \pm 0.2	2.7 \pm 1.3		1.8 \pm 0.4
Alanine	21.4 \pm 6.9	17.3 \pm 4.9		13.1 \pm 6.3
1/2-Cystine	...	0.8** \pm 0.1		0.5** \pm 0.1
Valine	1.3 \pm 0.2	12.1 \pm 3.7		8.4 \pm 3.2
Isoleucine	...	2.5 \pm 1.9		1.6 \pm 0.7
γ -Aminobutyric acid	4.5 \pm 3.7	8.4 \pm 3.2		4.7 \pm 0.9
U	...	1.8 \pm 0.4		1.4 \pm 0.5
Ammonia	50.8 \pm 17.3	81.8 \pm 25.6		48.5 \pm 6.2
Lysine	0.4	2.2 \pm 0.8		1.3 \pm 0.4
Histidine	...	1.7 \pm 0.1		1.3 \pm 0.2
Arginine	...	1.7 \pm 0.5		1.8 \pm 0.7
Totals	128.0	377.4		502.5

* Average and standard deviation of 4 analyses of common control shoots, 5 of all others. The shoots were each up to 15 cm long and weighed approximately 0.5 g.

** Three or 4 determinations only.

reached at 5 and 7 days without water, respectively. On these days separate plants of each variety were labeled with $200 \mu\text{C } ^{14}\text{CO}_2$ as described above. Separate shoots were excised from each of the labeled plants for measurement of free amino acids and of protein-bound amino acids at 1, 5, 12, 29, and 77 hours after the end of the labeling period. Fresh weight, dry weight, and water potential were measured on shoots from unlabeled duplicate plants every day during the 77-hour sampling period after labeling.

A summary of the measurements made is given in table I. During the 77-hour sampling period, water stress was increasing. The highest water potential measurement given was made at the time of labeling, and it decreased thereafter. Therefore all averages of measurements made during the sampling period apply to the entire period when water potential was changing, and not to any average water potential.

Total free amino acids doubled in Common and tripled in Coastal shoots (table I) at maximum

water stress employed. At the same time, water-soluble protein decreased in stressed shoots to less than half that of controls. The sum of free and protein-bound amino acids for each variety remained almost constant among all treatments.

The time course of changes in ^{14}C labeling of the total free and water-soluble protein-bound amino acids is shown in figure 2. Initial incorporation of ^{14}C was greatest into, and the most label was retained in, the free amino acid fraction from moderately stressed shoots. Incorporation of label into the free amino acid fraction of severely stressed shoots was not as great as for moderately stressed shoots, but again more label was retained than in controls. Greater treatment differences were found in the incorporation of label into protein. Controls accumulated label into protein continuously during the sampling period. Label in protein from moderately stressed shoots reached a maximum after 5 hours and declined thereafter. At the 77th hour the amount of label in protein from moderately-stressed shoots was only 20 % that

Table III. Effect of Water Stress on Protein Composition

Average and standard deviation of 4 measurements for Common control, 5 for all others. Cystine and tryptophan not determined and not included in calculations. Amide N not determined.

Amino acid	Control	Mole percent Moderate stress	Severe stress
Common			
Aspartic acid	10.1 \pm 1.5	9.0 \pm 1.2	9.1 \pm 1.1
Threonine	4.9 \pm 0.5	4.0 \pm 0.3	4.4 \pm 0.4
Serine	4.9 \pm 0.2	4.7 \pm 0.3	5.1 \pm 0.6
Glutamic acid	11.0 \pm 0.6	11.5 \pm 0.4	11.5 \pm 0.8
Proline	5.5 \pm 0.3	6.0 \pm 0.4	5.5 \pm 0.7
Glycine	9.7 \pm 0.4	11.0 \pm 0.7	11.0 \pm 0.7
Alanine	9.9 \pm 0.5	10.9 \pm 0.4	10.9 \pm 0.3
Valine	8.3 \pm 0.5	8.5 \pm 0.4	8.4 \pm 0.6
Methionine	1.4 \pm 0.6	1.6 \pm 0.6	1.7 \pm 0.3
Isoleucine	5.5 \pm 0.6	6.2 \pm 1.0	5.4 \pm 0.2
Leucine	9.1 \pm 0.6	9.6 \pm 0.4	9.3 \pm 0.5
Tyrosine	3.1 \pm 0.2	3.9 \pm 0.3	3.1 \pm 0.4
Phenylalanine	4.1 \pm 0.2	4.2 \pm 0.2	4.1 \pm 0.3
Lysine	6.3 \pm 0.5	5.7 \pm 0.3	6.2 \pm 0.8
Histidine	1.8 \pm 0.2	1.6 \pm 0.3	1.7 \pm 0.3
Arginine	4.5 \pm 0.5	2.7 \pm 0.6	3.1 \pm 0.5
Coastal			
Aspartic acid	9.2 \pm 0.5	9.2 \pm 0.9	9.7 \pm 0.5
Threonine	4.6 \pm 0.3	4.1 \pm 0.3	4.2 \pm 0.4
Serine	5.0 \pm 0.2	4.8 \pm 0.3	5.4 \pm 1.0
Glutamic acid	11.1 \pm 0.6	11.6 \pm 0.3	11.4 \pm 0.9
Proline	5.9 \pm 0.7	5.8 \pm 0.5	5.4 \pm 0.9
Glycine	9.9 \pm 0.4	11.1 \pm 0.5	10.7 \pm 0.5
Alanine	10.2 \pm 0.3	10.6 \pm 0.5	10.9 \pm 0.5
Valine	8.2 \pm 0.2	8.5 \pm 0.3	8.7 \pm 0.2
Methionine	1.7 \pm 0.4	1.9 \pm 0.1	1.6 \pm 0.5
Isoleucine	5.8 \pm 0.2	5.5 \pm 0.3	5.3 \pm 0.3
Leucine	9.3 \pm 0.2	9.6 \pm 0.3	9.1 \pm 0.7
Tyrosine	3.1 \pm 0.3	2.9 \pm 0.2	2.7 \pm 0.3
Phenylalanine	4.4 \pm 0.7	4.1 \pm 0.2	3.9 \pm 0.3
Lysine	6.0 \pm 0.8	5.6 \pm 0.5	6.2 \pm 0.9
Histidine	1.8 \pm 0.2	1.8 \pm 0.3	1.7 \pm 0.2
Arginine	3.9 \pm 0.6	2.9 \pm 0.5	3.1 \pm 0.5

BARNETT AND NAYLOR—AMINO ACID AND PROTEIN METABOLISM

1227

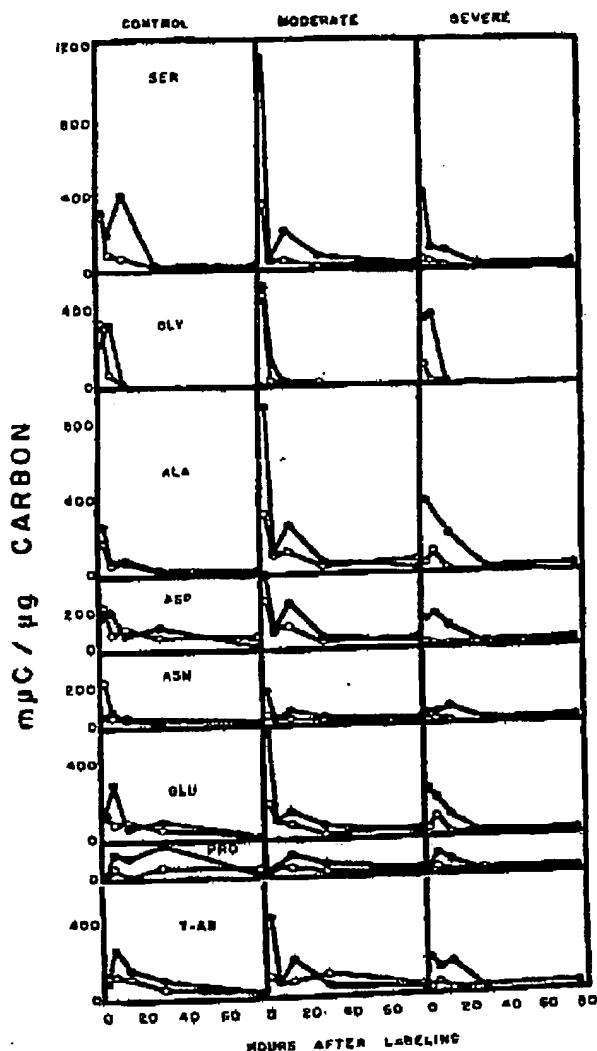


FIG. 3. Changes in specific activity of individual free amino acids from $^{14}\text{CO}_2$ -labeled Bermuda grass shoots.

of controls. Very little label was incorporated into protein from severely stressed shoots. These data support the conclusion that free amino acids are readily synthesized during water stress, but are not as readily incorporated into protein as in unstressed controls.

Changes in amounts of individual free amino acids with increasing water stress are shown in table II. The free amino acid concentrations in control shoots of both varieties were similar except that Common shoots contained more than twice as much free asparagine as Coastal shoots. From comparison of the blue-yellow light absorption ratios for the ninhydrin reaction products of asparagine and threonine, and from preliminary analyses of mild acid hydrolysates of purified free amino acid extracts, it was found that the asparagine content

of shoots is much greater than the threonine content. Therefore the combined asparagine-threonine peak was calculated as asparagine. The increase in this peak during water stress was also due to increased asparagine content. Free proline concentration increased dramatically during water stress to 10 to 125 times its control value. At the same time, valine content tripled, and glutamic acid and alanine concentrations decreased.

Serine, glycine, and alanine were the free amino acids that became most highly labeled (fig 3). Aspartic acid, glutamic acid, γ -aminobutyric acid, and asparagine all incorporated somewhat less label. All of the free amino acids found in the controls except proline became more highly labeled in the moderately stressed shoots than in any other treatment or the controls. Proline seemed to become labeled slowly. The low specific activity of proline obscures the fact that the great amount of proline present in stressed plants contained more than half the activity remaining in the free amino acid fraction after 77 hours. The irregularities in changes in specific activities of the individual amino acids occur for all the amino acids of a specific sample. This indicates that the differences reside in the level of labeling of individual shoots, and not that specific activities changed irregularly within any 1 shoot.

Changes in the amino acid composition of water-soluble protein with increasing water stress are given in table III. Conditions of hydrolysis did not permit preservation of cystine or tryptophan; consequently these had to be omitted from the calculations. The largest change in protein composition in stressed shoots was the 20 to 40 % decrease in arginine content. There was also a small decrease in threonine content.

Changes in the specific activity of individual protein-bound amino acids are shown in figure 4. The higher specific activity in Common shoots is attributed to the smaller size of Common shoots, which results in a concentration of label when plants were exposed to equal amounts of label. The specific activity curves for most protein amino acids from control or moderately stressed shoots level off after 5 or 12 hours. Specific activity of protein amino acids from severely stressed shoots was considerably lower than in the other treatments. Generally maximum specific activity was achieved after 1 to 12 hours, followed by a sharp decline. The specific activity curves, together with the protein data of table I, are interpreted to mean that there is a net loss of protein during water stress although some synthesis occurred.

To test whether or not proline could be synthesized from glutamic acid (17) in stressed plants, control and stressed shoots were incubated with glutamic acid- ^{14}C . Two well-watered control shoots and 2 shoots from a Coastal plant not watered for 6 days were excised and quickly placed in 0.05 ml of water contained in 2 cm conical centrifuge tube tips. The shoots were illuminated as before.

1228

PLANT PHYSIOLOGY

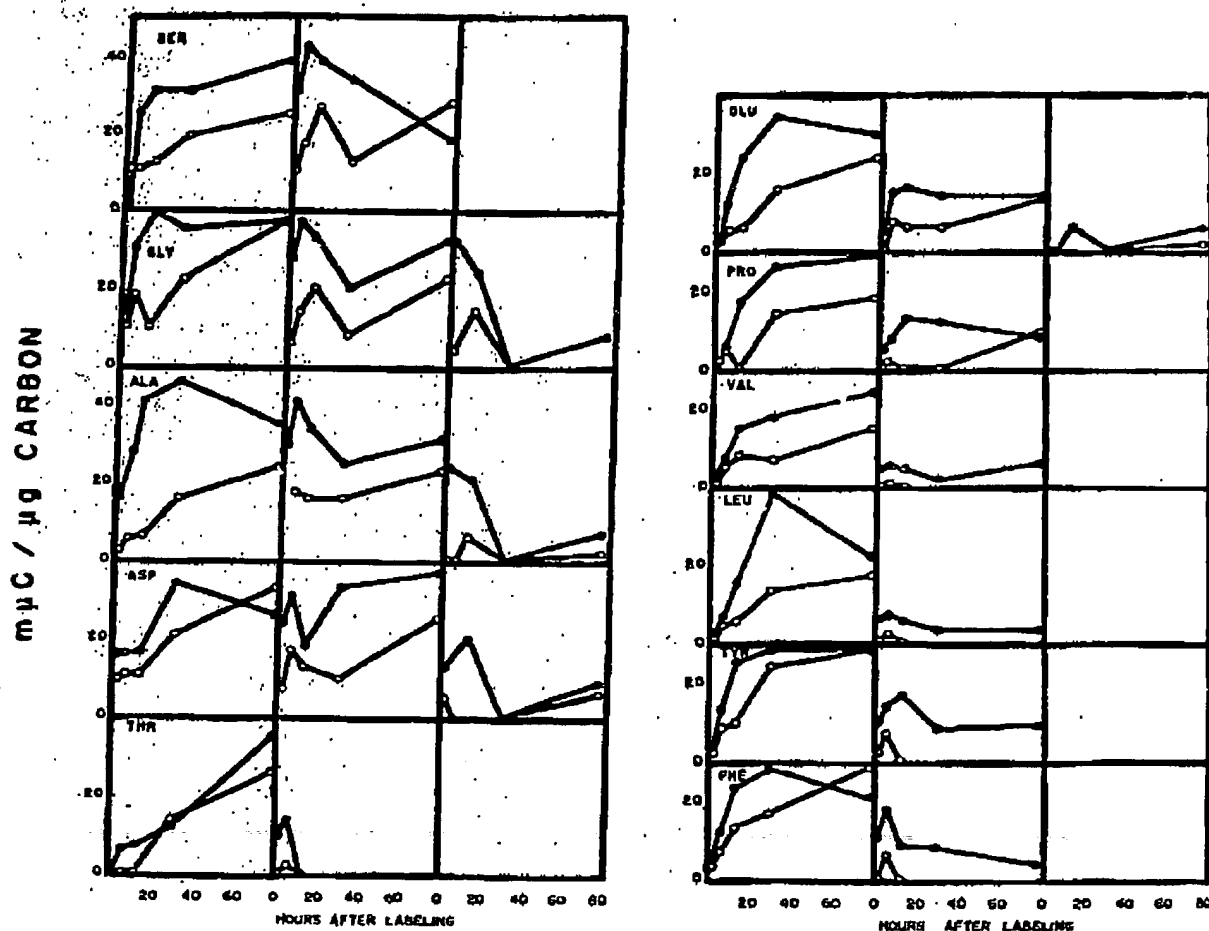


FIG. 4. Changes in specific activity of individual soluble protein amino acids from $^{14}\text{CO}_2$ -labeled Bermuda grass shoots. Specific activities were calculated from average protein composition values for Common Bermuda controls and the measured radioactivity figures.

To the water was added $3.0 \mu\text{C}$ of randomly labeled ^{14}C -glutamic acid, monoammonium salt, specific activity 10 mC mmole^{-1} . Water was added to the vessels in 0.02 ml increments to replace that taken up by the shoots. One control and 1 stressed shoot were killed in boiling 80% ethanol after 1 hour; the other 2 shoots were killed after 3 hours. Amino acid extracts were made in the usual manner, except that the ion exchange purification step was omitted. Radioactivity and ninhydrin-positive compounds were measured on the analyzer.

Results are shown in table IV. About half of the amino acids, plus at least 11 ninhydrin-negative compounds, became labeled both in control and stressed shoots. Proline was very slightly labeled in controls, but specific activity was fairly high because of the low amount present. The proportion of recovered label in proline from controls was less than 1% at both sampling times, whereas this pro-

portion in stressed plants was 6.0% at 1 hour and somewhat less than 8.6% at 3 hours. The actual activity in proline was 25 and 16 times greater in stressed plants than in controls at 1 and 3 hours respectively. It is concluded that water-stressed Bermuda grass shoots convert glutamic acid to proline, and accumulate the newly synthesized proline, much more readily than do well-watered shoots. Turnover of new proline was also very slow; labeled proline was still being accumulated 3 hours after labeling.

The data of table IV also show that glutamic acid- ^{14}C disappears at about equal rates from stressed and control shoots. The largest amounts of label were recovered in several ninhydrin-negative peaks eluted before aspartic acid. These peaks represent sugars and organic acids which would ordinarily have been lost if the sample had been purified by the ion exchange method.

BARNETT AND NAYLOR—AMINO ACID AND PROTEIN METABOLISM

1229

Table IV. Distribution of Radioactivity in Soluble Compounds of Coastal Bermuda Grass Shoots Incubated with Glutamic Acid- $U^{14}C$

Compound	Control (-3.5 bars)		Stressed (-25.2 bars)	
	1	3	1	3
1	67
2	71	194
3	53	170
4	83	250	...	173
5	83	101
6	120	53	...	80
7	77	53
8	11	14
9	12
10	6.5	2.4	...	5
11	10	3	...	67
12	73.5	31	...	34
Aspartic acid	19	7.1	14	15
Asparagine and threonine	15	16	12	192
Serine	268	188	410	101
Glutamic acid	2	6.5	50	...
Proline	2	...
Glycine	47	81
Alanine	77	28	...	1
12	8	5	...	28
13	40	11	35	...
Valine	1	2	...	1
Isoleucine	1	2	1	1
14	4	1
Leucine	3	...	2	2
15	2	...	1	12
γ -Aminobutyric acid	91.5	24	63	...
Totals	1130	854	790	1170

* Ninhydrin-negative compounds, numbered in order of elution from the analyzer column, were detected as radioactivity peaks only.

Discussion

Soluble protein levels in Bermuda grass were found to decrease with increasing water stress. Chen et al. (3) have reported successive increase, decrease, and a second increase in protein levels with increasing stress in citrus seedlings. These changes parallel Stocker's (15) activation, reaction, and restitution phases of drought response. The data presented here do not fit this pattern. However, the Bermuda grass data represents certain water stress levels, while the citrus data were taken at strict time intervals after withholding water.

The marked loss of protein-bound arginine in stressed Bermuda grass shoots has not been reported for other plants. This loss may reflect a preferential hydrolysis of arginine-rich protein. Such proteins are found in nuclei (5) and in ribosomes (16). Water stress can induce either an increase (19) or a decrease (14) in ribosomal RNA, but ribosomal proteins and nuclear proteins have not been investigated in connection with water stress. However, basic nuclear and ribosomal proteins as a whole are rich in lysine as well as in

arginine, and no such loss in protein bound lysine was detected as a result of water stress. This could be interpreted to mean the loss of protein arginine involves the loss of some arginine-rich but lysine-poor protein. Furthermore, the loss in protein arginine may account for the observed very slight rise in free arginine.

During severe water stress, photosynthesis, starch accumulation, and protein synthesis are all inhibited to some degree. In stressed Bermuda grass shoots enough $^{14}CO_2$ was fixed to label free proline that turned over very slowly. The ^{14}C -proline that turned over very slowly. The ^{14}C -glutamic acid labeling data clearly show that stressed shoots readily accumulated much more proline newly synthesized from glutamic acid than do control shoots. The slow turnover of labeled proline may also reflect an inhibition of proline catabolism. Free proline may be acting as a storage compound for both carbon and nitrogen during water stress, when both starch and protein synthesis are inhibited. Such a storage compound might be utilized for growth upon rewatering.

The changes in levels of free amino acids accompanying water stress in Bermuda grass are similar to those found in water stressed citrus

1230

PLANT PHYSIOLOGY

seedlings (3), pumpkin roots (20), and cut ryegrass (6).

Throughout this study, possible differences in nitrogen metabolism between Common and Coastal varieties were sought. During water stress, free proline accumulated to the highest levels in Coastal shoots. Under well watered conditions Common shoots contained the largest amounts of free asparagine. Aside from these minor observations, no differences were detected that might serve as a basis for explanation of the known differences in drought response. Such differences are still best explained on anatomical and morphological grounds (13).

Acknowledgments

We thank Dr. G. W. Burton, Tifton, Georgia, for clonal material of grasses, and Dr. P. J. Kramer for valuable advice and discussion.

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WGK

CHAPTER 9

Betaines

R. G. WYN JONES AND R. STOREY

I.	Introduction	172
A.	Definitions and nomenclature	172
B.	Naturally occurring betaines	175
II.	Phytotaxonomic distribution of betaines	176
A.	Glycinebetaine	176
B.	Prolinebetaine and hydroxyprolinebetaines	176
C.	β -alaninebetaine	187
D.	Trigonelline and homarine	187
E.	Other betaines	188
F.	Related ammonio compounds	188
G.	Related sulphonio compounds	189
III.	Tissue distribution of betaines	190
A.	Vegetative tissues	190
B.	Non-vegetative tissues	190
IV.	Biosynthetic pathways	191
A.	Glycinebetaine	191
B.	Prolinebetaine	193
C.	β -alaninebetaine	193
V.	Accumulation of betaines in stressed tissues	194
A.	Glycinebetaine	194
B.	Comparison of glycinebetaine and proline accumulation and consumption	196
C.	β -alaninebetaine and prolinebetaine	198
VI.	Possible physiological functions	200
A.	Glycinebetaine as a cytoplasmic osmoticum	200
B.	Other aspects of the physiological chemistry of glycinebetaine	202
C.	Possible function of β -alaninebetaine	203
VII.	Concluding discussion	204

1. INTRODUCTION

A. DEFINITIONS AND NOMENCLATURE

In 1953 Wheeland defined *onium* compounds as "substances formed by an addition reaction, in the course of which some atom increases its valency by one unit and in doing so increases its formal charge, algebraically, by one unit". This definition encompasses a very broad group of compounds of which quaternary ammonium and tertiary sulphonium compounds with fixed positive charges on, respectively, the nitrogen and sulphur atoms are probably the more important biologically. These are widely, but apparently erratically, distributed in nature. Cantoni (1960) noted their greater abundance and diversity in marine invertebrates, fish and certain plants compared with micro-organisms and vertebrates. Nevertheless, specific onium compounds, such as acetylcholine, have a fundamental role in vertebrate biochemistry.

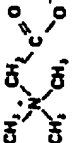
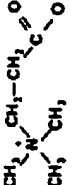
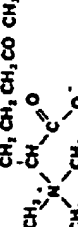
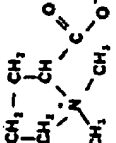
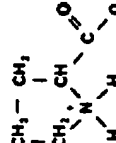
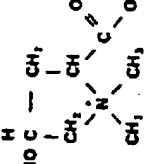
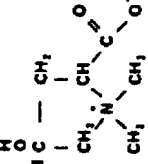
In this chapter our major concern will be *ammonio* compounds (the preferred chemical term), particularly that class of fully N-methyl substituted amino acids to which the trivial name *betaines* is applied. Some attention will also be directed to closely related compounds, for example choline-O-sulphate, and to *sulphonio* analogues of the betaines. In plant biochemistry there is some interest in phosphonium compounds in view of their role as plant growth regulators (Cathey, 1964), but they lie outside the scope of this chapter.

The trivial nomenclature in this field is in great disarray and, with the increasing interest in the physiology and biochemistry of these compounds, the establishment of a clear and agreed system assumes some importance. We will adopt the convention of naming individual betaines by reference to their parent amino acid (e.g. N,N'-dimethylproline, often referred to as stachydrine, will be called prolinebetaine by analogy with the well-established practice of calling N,N'-trimethylglycine glycinebetaine). A number of betaines found in plants, together with their trivial names, are recorded in Table 1. This system of naming will not be applied to the "betaines" produced by the methylation of the ring nitrogen of pyridine as there is no root amino acid; thus the name trigonelline will be retained (Table 1). We feel it is important that the use of "betaine" to describe glycinebetaine be discontinued, despite our own use of the term in early papers and its recent use by others (Hall et al., 1978; Hanson & Nelson, 1978). Using the same term for a class of compounds and for a particular member of that class can only lead to confusion. The term, *thetin*, for methyl-substituted sulphonio compounds will be dispensed with as it is not (as used by Challenger, 1959) formally analogous with "betaine"; the recognized chemical terminology, sulphonium (sulphonio), will be employed.

9. Betaines

TABLE 1

Names and structures of major betaines.

Structures	Preferred trivial names	Other trivial names
	Glycinebetaine	Oxycoccin, Betaine
	β -alaninebetaine	Homobetaine
	2-trimethylamino-6-ketohexanoate	
	Prolinebetaine	Stachydrine
	Proline	
	(-)-14-hydroxyprolinebetaine	Betocaine
	(+)-14-hydroxyprolinebetaine	Turcine

B. NATURALLY OCCURRING BETAINES

Of the wide variety of betaines found in plants, glycinebetaine was the first to be isolated and has been subjected to the closest scrutiny. The compound was first isolated by Husemann and Marme in 1863 from the arid zone shrub, *Lycium barbarum*, and was named lycin (see Karrer, 1958). Happily, this and other names found in the early literature (e.g. oxynurine, glykokollbetaine) have been abandoned. At the turn of the century, the extensive studies of Schulze and Stanek and their colleagues (see Guggenheim, 1958, for references) established that glycinebetaine was found in high concentrations in some plants, particularly *Atriplex* spp. and sugar beet. During this period prolinebetaine and trigonelline were also characterized, as were most of the more obscure betaines noted in Table I. β -alaninebetaine, however, was characterized relatively recently. Interest in sulphonio compounds developed rather later, and the work is described in Challenger's classic book (1959) on organic sulphur chemistry.

Despite the early activity of the German school of natural product organic chemists, little biochemical interest developed in these compounds apart from the recognition of the role of some N-methyl and S-methyl compounds in l-carbon metabolism and methyl donation (Cantoni, 1960). Only recently has an hypothesis been formulated to account for the large quantities of betaines, particularly glycinebetaine, found in certain plants (Storey & Wyn Jones, 1975; Wyn Jones et al., 1977a, 1977b). Glycinebetaine is proposed to be a major cytoplasmic osmoticum in certain higher plant families adapted to salt or water stress. It may be that other betaines and, possibly, methyl sulphonio compounds have a similar function in other species, but in all cases inadequate evidence is available.

In this chapter we will summarize data on the taxonomic and tissue distribution of betaines and will consider their biochemistry and physiology in relation to water and salt stress. It is inevitable that the major emphasis will be on glycinebetaine as less is known about the other compounds. And it is of particular interest to consider whether glycinebetaine accumulation is a positive adaptation to stress or an incidental side reaction. There is extensive literature on betaines and related compounds in invertebrates and, to a lesser extent, marine algae, besides the work of Avi-Dor and his colleagues on a moderately halophilic bacterium.

Table I continued
Names and Structures of major betaines.

Structures	Preferred trivial names	Other trivial names
	3-hydroxymethylbetaine	3-oxysaccharine
	Histidinebetaine	Herrysaline Excitine
	Tryptophanbetaine	Hypophorine
	2-mercaptobiosidobetaine	Ergothioneine
	Pipicolobetaine	Homosaccharine
	Trigonelline	

II. PHYTOTAXONOMIC DISTRIBUTION OF BETAINES

A. GLYCINEBETAINES

Glycinebetaine is structurally the simplest of the betaines; its dipolar structure is given in Table I. Proline is also included in Table I to illustrate the structural similarities of some of the compounds under discussion. As far as is known, glycinebetaine is always present as the free dipole, but this is not the case for some of the other betaines, notably β -alaninebetaine.

In Table II data on the taxonomic distribution of glycinebetaine and some of the other betaines are summarized. Glycinebetaine is widely distributed and often occurs in very large quantities. The data suggest that there is a taxonomic basis to glycinebetaine distribution and almost certainly to its accumulation as a major metabolite. Much more work will be required to establish this firmly and to define which families and species have this capacity.

There are indications in Table II that glycinebetaine accumulation is associated with halophytic and, possibly, xerophytic characteristics and arid and salty habitats. There is, however, a certain distortion inherent in these data. Because glycinebetaine was found in halophytic species, there has been a tendency to concentrate the various surveys on saline and dry habitats, and relatively few glycophytes and mesophytes have been studied. Not all xerophytic or halophytic species accumulate glycinebetaine or other betaines under field conditions, and no salt-sensitive or drought-sensitive plant with a low sap osmotic pressure has been found to accumulate one of these compounds.

Glycinebetaine has also been found in considerable concentrations in the tissues (usually muscle) of marine invertebrates (Beers, 1967; Schoffeniels & Gilles, 1972), in fungi (Ardreva, 1971), in some marine algae (Takemoto & Sai, 1964), and in bacteria (Rafaeli-Eshkol & Avi-Dor, 1968).

B. PROLINEBETAINES AND HYDROXYPROLINEBETAINES

Much attention is focused in this volume on the physiology and biochemistry of proline accumulation in plants subject to water stress. Consequently, it is of particular interest that the betaines of proline and of 3- and 4-hydroxyproline occur in plants (see Table I for nomenclature and structures; see Table II for phytochemical distribution). Of these compounds, prolinebetaine itself appears the most common and is a characteristic product

9. Betaines

DICOTYLEDONS ^a				
Genus and species	Tissue	Osmotic potential (10 ³ Pa)	Name	Onium compound $\mu\text{mol} \cdot 8 \text{ dry wt}^{-1}$ or (g fresh wt ⁻¹)
Order Capparales				
FAMILY CAPPARACEAE				
36 species	leaves		pb	
<i>Capparis tomentosa</i>	seeds		OH-pb	
Order Euphorbiales				
FAMILY EUPHORBACEAE				
	leaves	-8.6	trig	
<i>Euphorbia paralias</i>				
Order Leguminales				
FAMILY LEGUMINOSAE				
shoots		-27.0	ND	arid sandy
<i>Acacia hakeoides</i>	leaves	-22.5	ND	arid sandy
<i>Acacia lineata</i>	shoots	-24.4	ND	saline
<i>Acacia senophylla</i>	shoots	-25.3	ND	arid sandy
<i>Acacia tripartita</i>	shoots	-21.2	trig	arid sandy
<i>Cassia artemisioides</i>	shoots	-21.0	pb	arid sandy
<i>Hedicago sativa</i>	shoots	-11.3	trig	grown in 200 mmol NaCl
<i>Phaseolus vulgaris</i>	shoots	-18.2	trig	grown in 75 mmol NaCl
<i>Platanus sativus</i>	shoots	-11.3	trig	grown in 100 mmol NaCl
<i>Trifolium repens</i>	shoots	-11.3	trig	grown in 75 mmol NaCl
<i>Vicia faba</i>	shoots	-11.3	trig	grown in 75 mmol NaCl
Distribution of betaines in higher plants				
4Q	4Q	present in 33 species		arid zone
4.5 V				
1 A				coastal sand dune
3 H				
2 B				
2 B				
2 B				
2 B				
12				
1 C D				
1 C D				
1 C D				

Table II continued

Order Malvales						
FAMILY MALVACEAE						
<i>Abutilon albescens</i>	leaves	-1.6	gb	213	arid coral island	2 D
<i>Abutilon otocarpum</i>	leaves	-36.1	gb		halophyte	2 B
<i>Lavatera plebeja</i>	shoots	-19.8	D+		arid sandy	2 B
Order Myrtales						
FAMILY MYRTACEAE						
<i>Melaleuca ucinata</i>	shoots	-25.0	D+		arid sandy	2 B
Order Sapindales						
FAMILY SAPINDACEAE						
<i>Dodonaea attenuata</i>	shoots	-23.9	ND		saline	2 B
<i>Heterodendron oleifolium</i>	shoots	-20.2	trig		arid sandy	2 B
Order Verbenales						
FAMILY VERBENACEAE						
<i>Avicennia marina</i>	leaves	-33.8	gb	265	intertidal mangrove	2 B

DICOTYLEDONS
Division II Herbaceae (fundamentally herbaceous)

Genus and species	Tissue	Osmotic potential (10 ⁵ Pa)	Name	Onium compound $\mu\text{mol. g dry wt}^{-1}$ or (g fresh wt^{-1})	Ecology or habitat of collected species	Assay method and reference
Order Asterales						
FAMILY ASTERACEAE (Composite)						
<i>Aster tripolium</i>	shoots	-24.9	gb	164	salt marsh	1 A
	shoots		gb.(P)	(29)	coastal	W
<i>Centaurea melitensis</i>	shoots	-15.4	ND		arid sandy	2 B
<i>Chondrilla juncea</i>	shoots	-14.4	ND		arid sandy	2 B
<i>Erigeron bonariensis</i>	shoots	-19.2	gb	68	salt marsh	2 B

<i>Helichrysum apiculatum</i>	shoots	-16.4	ND			
<i>Ixiolaena leptolepis</i>	shoots	-27.2	ND		arid sandy	2 B
<i>Marricaria maritima</i>	shoots	-6.4	gb, D+		arid saline	2 B
<i>Minuria leptophylla</i>	shoots	-50.6	gb	55	coastal sand dune	1 A
<i>Myriocephalus ruarui</i>	shoots	-10.0	ND	175	halophyte	2 B
<i>Pluchea lanceolata</i>			gb		arid sandy	2 B
<i>Senecio spathulatus</i>	shoots	-15.3	pb		hot regions of India	4 I
<i>Sonchus oleraceus</i>	shoots	-21.2	ND		coastal sand dune	2 F
<i>Vitadina cuneata</i>	shoots	-17.2	gb		dry saline	2 B
<i>Waitzia acuminata</i>	shoots	-15.9	ND	42	arid sandy	2 B
<i>Wedelia biflora</i>	leaves	-9.3	dsp		dry saline	2 B
					dry coral island	2 E
Order Brassicales						
FAMILY CRUCIFERAE						
<i>Cakile maritima</i>	shoots	-14.9	ND		sand dune	1 A
<i>Cochlearia officinalis</i>	shoots		gb.(P)	trace	coastal	W
<i>Raphanus sativa</i>	shoots		ND		grown in 100 mmol NaCl	2 D
Order Caryophyllales						
FAMILY AIZOACEAE						
<i>Mesembryanthemum crystallinum</i>	shoots		ND			
<i>Psilocaulon</i> (Haw) Schwautes	shoots	-38.3	2 D+		200 mmol NaCl	2 Z
<i>Tetragonia expansa</i>	tops		ND		saline	2 B
FAMILY CAROPHYLLACEAE						
<i>Spergularia marina</i>	shoots		gb.(P)	trace	coastal	5 J
<i>Spergularia media</i>	shoots		gb.(P)	trace	coastal	W
Order Chenopodiales						
FAMILY CHENOPODIACEAE						
<i>Arthrocnemum halocnemoides</i>	shoots	-59.1	gb	280	halophyte	2 B
<i>Atriplex canescens</i>	leaves		gb	279	halophyte	3 H
	leaves		gb	323	halophyte	5 J
<i>Atriplex halimus</i>	leaves		gb	418	halophyte	3 H
<i>Atriplex hastata</i>	shoots	-18.1	gb	75	halophyte	2 B

R. G. W. J. Jones and R. H. Hickey

a. Melitensis

179

Table II continued

<i>Atriplex hortensis</i>			gb	84-107		3 H
<i>Atriplex inflata</i>	shoots	-34.7	gb	223	halophyte	2 B
<i>Atriplex nummularia</i>	leaves	-37.7	gb	152	halophyte	2 B
<i>Atriplex patula</i>	leaves		gb	112-225	halophyte	3 H
<i>Atriplex pseudocomplanata</i>	shoots	-40.2	gb	236	halophyte	2 B
<i>Atriplex rosea</i>	shoots		gb	94		5 J
<i>Atriplex semibaccata</i>	shoots	-35.6	gb	249	halophyte	2 B
<i>Atriplex spongiosa</i>	leaves	-34.7	gb	340	halophyte — grown in 500 mmol NaCl	2 C,D
<i>Atriplex suberecta</i>	leaves	-13.9	gb	260	saline	2 B
<i>Atriplex vesicaria</i>	shoots	-62.0	gb	251	halophyte	2 B
<i>Atriplex wootonii</i>	leaves		gb	284		5 J
<i>Babbagia actoptera</i>	shoots	-43.6	gb	187	halophyte	2 B
<i>Bassia brachyptera</i>	shoots	-43.2	gb	179	halophyte	2 B
<i>Bassia intricata</i>	shoots	-48.1	gb	214	halophyte	2 B
<i>Bassia lanicuspis</i>	shoots	-31.2	gb	272	halophyte	2 B
<i>Bassia parviflora</i>	shoots	-27.1	gb	169	halophyte	2 B
<i>Bassia patentiscuspis</i>	shoots	-31.3	gb	168	halophyte	2 B
<i>Bassia quinquecuspis</i>	shoots	-32.0	gb	171	halophyte	2 B
<i>Bassia stelligera</i>	shoots	-22.3	gb	193	arid saline	2 B
<i>Bassia tricuspid</i>	shoots	-51.9	gb	205	halophyte	2 B
<i>Beta cyclo</i>	leaves		gb	287		5 J
<i>Beta maritima</i>	leaves		gb	195	salt marsh	5 J
<i>Beta trigyna</i>	leaves		gb	179		5 J
<i>Beta vulgaris</i>	leaves		gb	223	salt resistant	5 J
<i>Chenopodium album</i>	leaves		gb	104		5 J
<i>Chenopodium bonus-henricus</i>	leaves		gb	190		3 H
<i>Chenopodium batrys</i>			gb	129		4 K
<i>Chenopodium foetidum</i>	whole plant		gb	123	sea-shore	5 J
<i>Chenopodium nitriaceum</i>	shoots	-32.9	gb	173	halophyte	2 B
<i>Chenopodium pseudomicrophyllum</i>	shoots	-33.4	gb	92	halophyte	2 B
<i>Chenopodium urticum</i>	shoots		gb	37		5 J
<i>Chenopodium vulvaria</i>	leaves		gb	158		3 H
<i>Corispermum marschellii</i>	whole plant		gb	97		5 J
<i>Corispermum tannoides</i>	shoots		gb	12		5 J
<i>Enchylaena tomentosa</i>	shoots		gb	35		5 J
<i>Habluzia tannoides</i>	shoots	-31.0	gb	216	halophyte	2 B
<i>Halimione portulacoides</i>	whole plant		gb	85		5 J
<i>Kochia trichophylla</i>	shoots	-20.5	gb	238	frost resistant salt marsh	1 A
<i>Kochia scoparia</i>	whole plant		gb	81		5 J
<i>Maireana sp.</i>	whole plant		gb	189		5 J
<i>Maireana appressa</i>	shoots	-28.8	gb	323	halophyte	2 B
<i>Maireana pyramidalis</i>	shoots	-39.4	gb	269	halophyte	2 B
<i>Malacocera tricornis</i>	shoots	-31.8	gb	278	halophyte	2 B
<i>Orbione sibirica</i>	shoots	-42.7	gb	36	halophyte	2 B
<i>Pachycornia tenuis</i>	shoots		gb	153	salt marsh	5 J
<i>Rhagodia spinescens</i>	shoots	-59.2	gb	533	halophyte	2 B
<i>Salicornia quinqueflora</i>	shoots	-32.3	gb	152	halophyte	2 B
<i>Salicornia frutescens</i>	shoots	-30.7	gb	181	wet saline	2 B
<i>Salicornia europaea</i>	shoots		gb	174	wet saline	4 L
<i>Salsola kali</i>	shoots		gb	(45)	coastal	W
<i>Salsola kali, tetrandra</i>	shoots		d	81	coastal dunes	5 J
<i>longifolia and rigida</i>				present in all 4		4 M
<i>Spinacia oleracea</i>	leaves		gb	23	salt resistant	3 H
<i>Suaeda maritima</i>	leaves		gb	162	salt resistant	5 J
<i>Suaeda monoica</i>	shoots		gb	(63)	wet saline	2 W,X
<i>Threlkeldia salsuginosa</i>	leaves	-53.1	gb	340	wet saline	2 C,D
FAMILY AMARANTHACEAE	shoots	-29.7	gb	203	halophyte	2 B
<i>Achyranthes aspera</i>	whole plant		gb	22		6 N
<i>Achyranthes aspera</i>	shoots	-7.0	gb			2 E
<i>Amaranthus caudatus</i>	leaves		gb	46	dry coral island	3 H
<i>Amaranthus caudatus</i>	leaves		gb	186		5 J
<i>Amaranthus retroflexus</i>	whole plant		gb	82		5 J

Table II continued

Order Lamiales						
FAMILY LABIATAE						
<i>Ajuga australis</i>	shoots	-14.1	ND		arid sandy	2 B
<i>Eremostachys speciosa</i>			pb	25		O
<i>Lamium album</i>			pb	140		O
<i>Lagochilus hirtus</i>			pb	57		3,4 P
<i>Lagochilus inebrians</i>			pb	36		O
<i>Lagochilus platycalyx</i>			pb	101		O
<i>Lagochilus pubescens</i>			pb	50		O
<i>Lagochilus setulosus</i>			pb	91		O
<i>Leonurus turkestanicus</i>			pb	105		O
<i>Marrubium vulgare</i>			OH-pb	19		6 Q
<i>Marrubium vulgare</i>	shoots	-18.4	D+		arid sandy	2 B
<i>Sideritis montana</i>			pb	57		O
<i>Stachys betonicaeflora</i>			pb	52		O
<i>Stachys hissarica</i>			pb	33		O
FAMILY MYOPORACEAE						
<i>Eremophila mitchelli</i>	shoots	-19.3	D+		arid sandy	2 B
Order Polygonales						
FAMILY POLYGONACEAE						
<i>Muehlenbeckia cunninghamii</i>	shoots	-22.1	trig		arid saline	2 B
<i>Polygonum aviculare</i>	shoots		ND			5 J
<i>Polygonum divaricatum</i>	shoots		ND			5 J
Order Plantaginales						
FAMILY PLANTAGINACEAE						
<i>Plantago coronopus</i>	shoots		ND		coastal	W
<i>Plantago maritima</i>	shoots		ND		coastal	1 A, W
Order Primulales						
FAMILY PLUMBAGINACEAE						
<i>Armeria maritima</i>	shoots	-19.8	β -ab.(P)		salt marsh	1 A, Y

R.C. Wyn Jones and R. Storey

<i>Limnium vulgare</i>	shoots	-24.7	β -ab.(P)		salt marsh	1 A
<i>Limnium vulgare</i>			β -ab.(P),mkh	50	halophyte	4,6 R, S
<i>Plumbago capensis</i>	shoots		ND			Y
FAMILY PRIMULACEAE						
<i>Glaux maritima</i>	shoots		gb.(P)	trace	coastal	W
Order Solanales						
FAMILY CONVOLVULACEAE						
<i>Evolvulus alsinoides</i>	whole plant		gb			4,6 T
FAMILY SOLANACEAE						
<i>Cestrum parqui</i>	leaves	-13.3	D+		arid sandy	2 B
<i>Lycium barbarum</i>	whole plant		gb	333		5 J
<i>Lycium chinense</i>	leaves		gb	166		3 H
<i>Lycium ferocissimum</i>	leaves	-24.5	gb	150	saline	2 B
<i>Lycopersicon esculentum</i>	leaves		trig	0.5	100 mmol NaCl	1 C, D
<i>Nicotiana velutina</i>	leaves	-15.3	ND		saline	2 B
<i>Solanum esuriale</i>	shoots	-16.1	trig		arid sandy	2 B
Order Umbellales						
FAMILY UMBELLIFERAE						
<i>Daucus carota</i>	shoots	-18.1	ND		100 mmol NaCl	1 D
<i>Eryngium maritimum</i>	shoots		ND		coastal	W

9. Bellines

MONOCOTYLEDONS

Genus and species	Tissue	Osmotic potential (10 ⁵ Pa)	Name	Onium compound μ mol. g dry wt ⁻¹ or (.g fresh wt ⁻¹)	Ecology or habitat of collected species	Assay method and reference
Order Graminales						
FAMILY GRAMINEAE						
Festuceae (Tribe)						
<i>Festuca rubra</i>	shoots		gb.(P)	(19)	coastal	W
<i>Puccinellia distans</i>	shoots		gb.(P)	trace	coastal	W

183

Table II continued

<i>Puccinellia maritima</i>	young leaves	-22.7	gb,(P)	2.6	salt marsh	1 A,W
Agrostaceae						
<i>Agrostis stolonifera</i>	*shoots		gb,(P)	(15)	coastal	W
<i>Ammophila arenaria</i>	shoots	-8.1	gb	113,(70)	coastal dunes	1 A,W
Hordeae						
<i>Agropyron junceiforme</i>	shoots		gb	(23)	coastal	W
<i>Agropyron pungens</i>	shoots		gb	(80)	coastal	W
<i>Triticum vulgare</i>	shoots	-18.1	gb	64	grown in 100 mmol NaCl	1 D
<i>Elymus arenaria</i>	shoots		gb	(77)	coastal	W
<i>Hordeum vulgare</i>	shoots	-15 - 17	gb	30 - 80	grown in 150 mmol NaCl	1 D
Aveneae						
<i>Avena sativa</i>	shoots	-17	gb	18	grown in 100 mmol NaCl	1 D
<i>Danthonia caespitosa</i>	shoots	-27	gb		saline	2 B
Chlorideae						
<i>Chloris acicularis</i>	shoots	-37	gb	85	halophyte	2 B
<i>Chloris gayana</i>	shoots		gb	4	grown in 150 mmol NaCl	1 D
<i>Spartina anglica</i>	shoots		gb,dsp,(P)	(120)	coastal	W
<i>Spartina townsendii</i>	shoots	-25	gb,dsp,(P)	258	salt marsh	1 A
Eragrostaceae						
<i>Diplachne fusca</i>	shoots		gb	40	saline gravel	1 U
Lepturaceae						
<i>Lepturus repens</i>	shoots	-8.5	gb		arid coral island	2 E
Sporoboleae						
<i>Sporobolus virginicus</i>	shoots	-20	gb	101	salt marsh	2 B
Panicaceae						
<i>Spinifex hirsutus</i>	shoots	-12	gb		coastal sand dune	2 F
Maydeae						
<i>Zea mays</i>	shoots	-13	gb	7	grown in 100 mmol NaCl	1 D
Zoysieae						
<i>Zoysia macrantha</i>	shoots	-15.5	gb	26	salt marsh	2 B
Uncertain						

<i>Amphipogon caricinus</i>	shoots	-22	gb		saline	2 B
<i>Sternotaphrum secundatum</i>	shoots	-13	gb	75	salt marsh	2 B
<i>Triodia irritans</i>	shoots	-28	gb	61	arid	2 B
Order Liliales						
FAMILY LILIACEAE						
<i>Asphodelus fistulosus</i>	shoots	-14.5	ND		saline	2 B
<i>Dianella revoluta</i>	shoots	-14	trig		arid sandy	2 B
Order Cyperales						
FAMILY CYPERACEAE						
<i>Carex arenaria</i>	shoots		ND		coastal	W
<i>Scirpus maritimus</i>	shoots		ND		coastal	W
Order Juncales						
FAMILY JUNCACEAE						
<i>Juncus gerardii</i>	shoots		ND		coastal	W
<i>Juncus maritimus</i>	shoots		ND		coastal	W
Order Aponogetonales						
FAMILY ZOSTERACEAE						
<i>Zostera marina</i>	shoots	-26	ND,(P)			2 Z
Order Juncaginiales						
FAMILY JUNCAGINACEAE						
<i>Triglochin maritima</i>	shoots	-23	ND,(P)		salt marsh	1 A,W
<i>Posidonia sp</i>	leaves	-26	dsp			2 Z

*Classification according to Hutchinson (1973).

9. Betaines

187

of at least two families, the Labiatae and the Capparidaceae. The hydroxyprolinebetaines are also found in these families. Prolinebetaine has also been reported in *Chrysanthemum* (Compositae), various *Citrus* (Rutaceae) and alfalfa (*Medicago sativa*, Leguminosae) (see Guggenheim, 1958, for early references). It is difficult with the evidence available to associate the prolinebetaine-accumulating families with particular ecological habitats. Some members of the Capparidaceae, for example *Capparis* spp., are found in hot arid regions (Walter, 1971) and indeed, Cornforth and Henry (1952) originally investigated *Capparis tomentosa* because of its reputed toxicity to camels! However, this cannot be said for the Labiatae, many of which, for example *Stachys* spp., occur in temperate countries.

The distribution of quaternary ammonium compounds in the Capparidaceae (Capparaceae), 39 species from 20 genera, have been studied by McLean (1976), who found prolinebetaine and/or hydroxyprolinebetaine in all but 4 genera. Surprisingly, while 12 genera contained choline salts 5 contained 3-hydroxy-1,1-dimethyl pyrrolidinium salts. Trace quantities of glycinebetaine were found in some species.

C. β -ALANINEBETAINE

Although β -alaninebetaine was originally identified by Engeland (1909), it was only detected in plants in 1973, in the marine alga *Monostroma nitidum* (Abe & Kaneda, 1973). More recently it has been found in a higher plant, *Limonium vulgare* (Larher & Hamelin, 1975a). In this plant β -alaninebetaine is a major nitrogenous component and may exceed 100 μ mol.g dry wt⁻¹ (Larher, 1976). A second ammonio compound has been found in *Limonium vulgare* — 2-trimethylamino-6-ketoheptanoate (Table 1) (Larher & Hamelin, 1975b). This compound was quantitatively far less important than β -alaninebetaine and occurred exclusively as the choline ester. Larher and Hamelin also reported that, in part, β -alaninebetaine occurs *in vivo* as the choline ester. As such, it would not be dipolar but would be a polycation with fixed positive charges at either end of the carbon ester chain.

So far only xerohalophytic members of the Plumbaginaceae have been reported to accumulate β -alaninebetaine. However, it remains to be seen if this rather specific distribution in higher plants is confirmed.

D. TRIGONELLINE AND HOMARINE

In a study of more than 100 plant species Klein et al. (1931) found trigonelline to be widely distributed in many families, including the Leguminosae, Solanaceae, Cruciferae, Compositae and the Gramineae. In many cases it occurs in the presence of a second, quantitatively more

- A. Storey et al. (1977)
B. Storey (unpublished data)
C. Storey (1976)
D. Storey and Wyn Jones (1977)
E. Pittman and Storey (unpublished data)
F. Condon and Storey (unpublished data)
G. Delaveau et al. (1973)
H. Cornwell and Rennie (1953)
I. Dasgupta et al. (1968)
J. Stanek and Domini (1909)
K. Ruzsitskova et al. (1973)
L. Suspluzes et al. (1969)
M. Karawa et al. (1971)
N. Kapoor and Singh (1966)
O. Pulatova (1969)
P. Proskurnina and Ustin (1960)
Q. Paulder and Wagner (1963)
R. Larher and Hamelin (1975a)
S. Larher and Hamelin (1975b)
T. Baveja and Singla (1969)
U. Sandhu et al. (1981)
V. Cornforth and Henry (1952)
W. Stewart et al. (1979)
X. Flowers and Hall (1978)
Y. Larher (1976)
Z. Wyn Jones unpublished data

1. Thin layer chromatography and periodide colorimetry
2. Ammonium rethickate or phosphotungstic acid precipitation
3. Chromatography and melting point determination
4. Periodide precipitation
5. Spectroscopy e.g. UV, IR, NMR or Mass
6. Not detectable
ND
gb Glycinebetaine
trig Trigonelline
D+ Unknown Dragendorff positive
Bab β -alaninebetaine
pb Prolinebetaine
OH-pb Hydroxyprolinebetaine
dsp Dimethylsulphonio propionate
(P) Significant proline levels found (> 15 μ mol.g fresh wt⁻¹)
tmk 2-trimethylamino-6-ketoheptanoate

Table 11 continued

9. Betaines

189

application increases the salt [5]. Danwitz et al., (1964) and drought (Halevy & Kessler, 1963; Larner et al., 1965) tolerance of plants, but these have been disputed (cf. Robertson & Greenway, 1973; Imbamba, 1973).

G. RELATED SULPHONIO COMPOUNDS

The sulphonio analogues appear to be less common and less various than their ammonio counterparts; nevertheless, they have rather similar distributions. Challenger and Simpson (1948) isolated β -dimethylsulphonioacetate from a marine alga and later (see Challenger, 1959) found that this compound occurs quite widely in marine algae, for example, *Polysiphonia* spp., *Enteromorpha* spp., and *Ulva lactuca*. It has also been reported in two fresh water species, *Oedogonium* sp. and *Ulothrix* sp., (Challenger, 1959). Recently it has been reported in a higher plant, *Spartina* sp. (Larner et al., 1977), and we have also found sulphonio compounds in large quantities in a

TABLE III
Name and structures of some methylated sulphonio and other related compounds.

Structures	Trivial names
	β -dimethylsulphonioacetate (β -dimethylpropionethin)
	δ -dimethylsulphonioacetate
	Choline-O-sulphate
	Choline-O-phosphate (Phosphoryl choline)
	Chlorocholine chloride CCC. Cycocel

R.G. Wyn Jones and R. Storey

important betaine. However, unlike glycinebetaine, it is not accumulated to high levels in vegetative tissues, although Blaim (1962) found concentrations of greater than 80 $\mu\text{mol g dry wt}^{-1}$ in the seeds of a number of legumes. The latter literature has not been reported in higher plants, although it has been found quite widely in marine invertebrates, particularly the Crustacea (Beers, 1967).

E. OTHER BETAINES

Tryptophanbetaine (Table I) has been found in the seeds of *Erythrina hypaphysa* (Leguminosae), while histidinebetaine has been isolated from the fungi *Auricularia campetris* and *Bolletia edulis* (for references see Guggenheim, 1958). Other betaine-like derivatives such as 2-mercaptobistidinebetaine, usually called ergothionein, have been found in fungi (Table I). This is similar to the simpler alkaloids and, indeed, in some texts the betaines are considered as alkaloids. These compounds, including histidinebetaine, etc., have also been reported in marine invertebrates (Ackerman & List, 1958).

F. RELATED AMMONIO COMPOUNDS

Choline, the alcohol related to glycinebetaine, is ubiquitously found in plants (Klein & Zeller, 1930; Toyosawa & Nishimoto, 1967; Storey & Wyn Jones, 1977) because of its role as a precursor of the major membrane constituent, phosphatidylcholine (lecithin). The sulphate and phosphate esters of choline have also been found (Table III). Tolbert and Wiebe (1955) identified choline-O-phosphate as a major phosphatic compound in the sap of tomato and barley, possibly involved in phosphate transport. Later, Nissen and Benson (1961) found that choline-O-sulphate accounted for 5–15% of labelled sulphate in *Zea mays*, *Hordeum vulgare* and *Helianthus annuus*. More relevant is the observation by Benson and Atkinson (1968) that, of the salt-secreting mangroves, *Avicennia* and *Aegialitis* spp. formed choline-O-sulphate, while *Aegiceras corniculatum* and *Acanthus illicifolius* formed choline-O-phosphate in large quantities. They suggested that these compounds could be involved in salt transport.

Trimethylamine has been found in flowers, and Cromwell (1950) observed that the surface glands on the stems, leaves and perianth of *Chenopodium vulvaria* produced this amine, which probably arose from choline degradation. The trimethylamine oxide is widely found in fish and marine invertebrates (Prosser, 1973) and is important as an osmoticum.

Structurally related to these compounds is the plant growth regulator chlorocholine chloride (Table III). Various reports have suggested that its

marine angiosperm, *Posidonia* sp. (Wyn Jones & Hughes, unpublished data) and in *Wedelia biflora* (Corham & Storey, unpublished data). Other, more complex sulphonio compounds occur in higher plants (the reader is referred to Challenger's book). In addition, Lather and Hamelin (1979) have isolated 5-dimethylsulphonopentanoate from the flowers of *Diplotaxis tenuifolia* (Cruciferae).

III. TISSUE DISTRIBUTION OF BETAINES

A. VEGETATIVE TISSUES

Only in the case of glycinebetaine is extensive data on tissue distribution available, and most of our comments will be restricted to this compound. Both β -alaninebetaine (Lather, 1976) and glycinebetaine (Storey & Wyn Jones, 1977) are normally found in higher concentrations in shoot than in root tissues of mature plants. Cromwell and Rennie (1953) showed that the glycinebetaine content of *Beta vulgaris* leaves generally increases with age, reaching a maximum at flowering, but the root level declines. Indeed, in the young seedling, the root level exceeds that of the shoot. However, at any particular time the young, actively growing leaves contain much more glycinebetaine than mature, expanded leaves. These trends were confirmed in members of the Chenopodiaceae by Simenauer (1975), and by Stanek's original data (1916). Storey (unpublished data) also found levels of 30 $\mu\text{mol.g}$ fresh wt^{-1} in unstressed *Atriplex spongiosa* apices compared with about 5 $\mu\text{mol.g}$ fresh wt^{-1} in more mature tissue.

Recently, the distribution of glycinebetaine in barley throughout its life cycle has been studied by Ahmad (1978), and a similar pattern emerged. The less mature leaves have higher values, and these decrease as the plant age increases. There is some evidence of transfer from the flag leaf, which has high levels, to the developing grain, which also accumulates glycinebetaine as it matures.

B. NON-VEGETATIVE TISSUES

A number of papers have reported the occurrence of glycinebetaine, prolinebetaine (Comforth & Henry, 1952) and trigonelline (Blain, 1962) in seeds. For example, glycinebetaine has been reported in cotton seed (Pollock & Stevens, 1965), in quite remarkable quantity in beet seed (256 $\mu\text{mol.g}$ fresh

wt^{-1}), and in *Spartina x townsendii* seed (68 $\mu\text{mol.g}$ fresh wt^{-1}) (Storey, 1976). Chittenden et al. (1978) found very high glycinebetaine concentrations in the mature wheat aleurone and embryo tissues but not in the starchy endosperm. The glycinebetaine content of the aleurone layer is much greater than the total internal amino acid pool. The amount of glycinebetaine did not change significantly during the first days of germination, but both it and the free amino acid pools were dramatically decreased by gibberellic acid treatment after 4 d of germination. Pearce et al. (1976) examined wheat ear tissues and, in Table IV, the glycinebetaine contents, as calculated from their data, are shown. As with the embryo and aleurone, partially dehydrated, but viable tissues, appear to have high levels.

TABLE IV
Glycinebetaine contents of wheat ear tissues.^a

Tissue	Glycinebetaine ($\mu\text{mol.g}$ dry wt^{-1})
anthers	96
paleas	40
lemmas	27
glumes	19
rachis	14
grain	10
leaves	14

^aData calculated from Pearce et al. (1976).

IV. BIOSYNTHETIC PATHWAYS

A. GLYCINEBETAINE

Two pathways for the biosynthesis of glycinebetaine may readily be envisaged (Cromwell & Rennie, 1954a, 1954b; Delwiche & Bregoff, 1957): the sequential methylation of the parent amino acid, glycine, or the oxidation of choline, itself formed by the sequential methylation of ethanolamine. All the recent evidence favours the second pathway, and data showing incorporation of ^{14}C -choline into glycinebetaine have been obtained with wheat (Bowman & Rohringer, 1970), barley (Hanson & Nelson, 1978) and a number of chenopods (Cromwell & Rennie, 1954a; Delwiche & Bregoff, 1957) (Fig. 1). This is also the pathway prevalent in animals (Paxton & Mayr, 1962).

9. Betaines

with the microbial degradative pathway (Kortstee, 1970; Wyn Jones et al., 1973), the probable path is through the sequential demethylation of glycinebetaine to glycine.

B. PROLINEBETAINE

In their study of prolinebetaine biosynthesis in alfalfa seedlings, Wiehler and Marion (1958) found that 2-week-old plants could convert ornithine and glutamate to proline and, on the further addition of both methionine and folic acid, ^{14}C -methyl label from the methionine was isolated in prolinebetaine. Thus, a pathway involving the sequential methylation of proline was proposed. In a later paper Robertson and Marion (1960), while able to show the conversion of N-methylproline (hygric acid) to prolinebetaine, were unable to show the conversion of proline itself and expressed some reservations whether the simple pathway of sequential methylation was in fact correct.

While prolinebetaine is the major betaine in young alfalfa seedlings, trigonelline and piperolatebetaine are equally important in seeds. Robertson and Marion (1959) made the interesting suggestion that the biosynthesis of these other betaines may only be initiated after anthesis.

C. β -ALANINEBETAINE

By feeding ^3H - β -alanine and ^{14}C -methyl-labelled methionine to *Limonium vulgare* leaves, Larher (1976) found that β -alaninebetaine was probably synthesized by the sequential methylation of the parent amino acid, and that methionine could well be acting as the methyl donor (see Fig. 2). Again the enzymology is unknown. In this context it is interesting to note Challenger's (1959) speculations on the relationship between the presence of dimethylsulphonio propionate and β -alanine in many marine algae (Ericson & Carlson, 1953). He suggested that the decomposition of the sulphonic compounds to dimethylsulphide ($(\text{CH}_3)_2\text{S}$), acrylic acid ($\text{CH}_2=\text{CHCOOH}$) and a proton (H^+), which has been shown to be enzyme mediated (Cantoni & Anderson, 1956), could initially liberate $^+\text{CH}_3\text{CH}_2\text{COO}^-$, which would yield β -alanine, with ammonia. However, we are aware of no recent biochemical work to confirm this speculative link between β -alanine, or its betaine, and dimethylsulphonio propionate.

R.G. Wyn Jones and R. Storey

As noted by Hanson and Nelson (1978), the major precursors of glycinebetaine, glycine, serine and formate are all intermediates in the photorespiratory or photo-oxidative carbon cycle. No enzymological data are available for higher plants, and nothing is known of the presumed choline dehydrogenase (choline oxidase) or glycinebetaine-aldehyde dehydrogenase enzymes, despite attempts to isolate them from chenopod tissue (Speed, 1972). Hanson and Nelson (1978) pointed out that, during the sequential methylation steps, there is a net demand for reducing power, which, using formate as the donor, would approximate to an NADPH requirement four times the rate of glycinebetaine accumulation. It should also be noted that there is a net production of a proton per glycinebetaine accumulated (Fig. 1).

The catabolic pathway has not been studied in plants but, by analogy

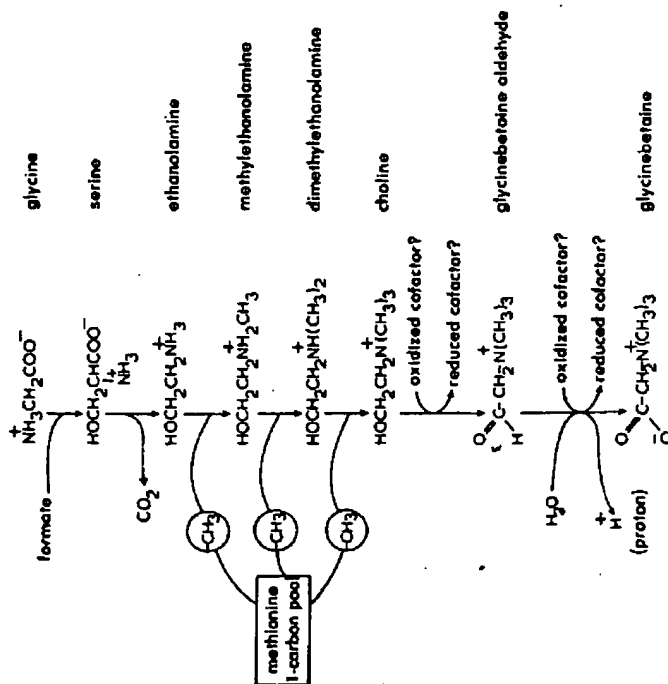


FIG. 1. Probable pathway for glycinebetaine biosynthesis.

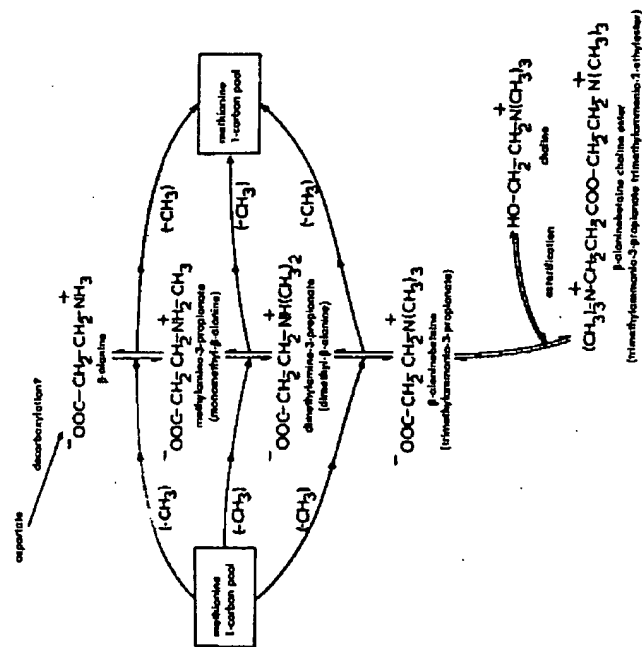


FIG. 2. Possible pathway for β-alanine betaine biosynthesis.

V. ACCUMULATION OF BETAINES IN STRESSED TISSUES

A. GLYCINEBETAINES

Although there was an intermittent interest in the distribution and biosynthesis of glycinebetaine in higher plants, and a few papers appeared describing the effects of added glycinebetaine on leaf disc and coleoptile expansion (Wheeler, 1963, 1965, 1969), little progress was made in exploring its function. However, this situation is changing rapidly following the key observation that the accumulation of the compound could be induced by salt or water stress (Storey & Wyn Jones, 1975; Storey, 1976). Evidence for progressive glycinebetaine accumulation in plants exposed to increasing

9. Betaines

195

external salt (NaCl) concentrations is now available for *Chloris gayana* (Storey & Wyn Jones, 1975), *Hordeum vulgare* cvs. *California Mariout* and *Arinar* (Wyn Jones & Storey, 1978a), *Diplachne fusca* (Sandhu et al., 1981), *Spartina x townsendii* (Storey & Wyn Jones, 1978b), *Atriplex spongiosa* and *Suaeda monoica* (Storey & Wyn Jones, 1979), and *Spinacia oleracea* (Coughlan & Wyn Jones, unpublished data).

Less complete but similar data are available for *Zea mays* var. *WF9 x M14*, *Triticum vulgare* var. *Capelle Desprez*, *Avena sativa* (Storey & Wyn Jones, 1977) and *Suaeda maritima* (Flowers & Hall, 1978), and there can be little doubt that external NaCl induces glycinebetaine accumulation in many, but perhaps not all members of the Gramineae and Chenopodiaceae (see *Puccinellia maritima* in Table II). However, the amounts accumulated vary from very small, in *Zea mays* (0.5–2 μmol/g fresh wt⁻¹), to very large, in some halophytic species (about 100 μmol/g fresh wt⁻¹), some of which have very high basal levels even in the absence of external salinity, for example *Suaeda monoica* with about 50 μmol/g fresh wt⁻¹ (Storey & Wyn Jones, 1975). In *Atriplex spongiosa* (Storey & Pitman, unpublished data) other salts, such as KCl, Na₂SO₄ and MgCl₂, also bring about glycinebetaine accumulation. However, glycinebetaine accumulation does not occur in salt-stressed *Atriplex spongiosa* plants when the relative humidity is maintained at 95–100%; these growth conditions do not result in the net salt accumulation in the leaves observed at lower relative humidities (Storey & Pitman, unpublished data).

There is less evidence to show that water stress brings about an increase in the glycinebetaine content of plants. Nevertheless, polyethylene glycol treatment and water withdrawal induces glycinebetaine accumulation in barley cultivars (Fig. 3; Hanson & Nelson, 1978), and similar observations have been made with *Spinacia oleracea* (Coughlan & Wyn Jones, 1980) and *Atriplex spongiosa* (Storey & Pitman, unpublished data). Thus, it is highly likely that accumulation can be brought about by both salt and water stress, although the former appears to be more effective. This may be due to the substantially greater osmotic pressure change in salt-stressed compared with water-stressed tissue. However, in marked contrast to proline accumulation (cf. Hsiao, 1973), glycinebetaine accumulation cannot be regarded as a general plant response to water stress, as many species, for example *Lycopersicon esculentum*, *Plum sativa*, *Daucus carota* (Storey & Wyn Jones, 1977), do not react in this manner. This difference is underlined by the phytotaxonomic data in Table II; glycinebetaine has not been detected in all plants having high sap osmotic pressures found in saline environments, or xeric environments.

Little is known of the biochemical mechanisms leading to the induction or activation of the glycinebetaine biosynthetic pathway. However it is

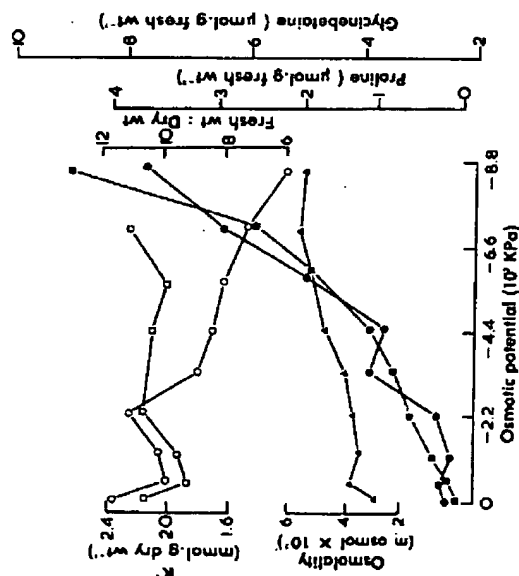


FIG. 3. Influence of water (polyethylene glycol) stress on glycinebetaine and proline levels and related parameters in barley, cv. California Marquis, subjected to a gradual incremental stress. (O) fresh wt. dry wt ratio; (Δ) osmolality; (■) glycinebetaine; (●) proline. (From Wyn Jones and Storey, 1978a.)

interesting to note that Lawlor and Fock (1978) found that the rate of photorespiration relative to photosynthesis increased in maize following water stress. Further, a greater proportion of ^{14}C from labelled CO_2 was found in the amino acids of the photorespiratory pathway, glycine and serine, both probable precursors of glycinebetaine (Fig. 1).

B. COMPARISON OF GLYCINEBETAINES AND PROLINE ACCUMULATION AND CONSUMPTION

The differences and similarities between glycinebetaine and proline accumulation in stressed plant tissues deserve further consideration. Hanson and Nelson (1978) and Wyn Jones and Storey (1978a) noted the close relation between the levels of these two solutes in barley subjected to water or salt stress. However, unstressed glycinebetaine levels are ten-fold higher than those of proline. Since a gradual stress induces a similar rate of glycinebetaine

9. Betaines

197

and proline accumulation (about $0.1\text{--}0.5\ \mu\text{mol}\cdot\text{g fresh wt}^{-1}\cdot\text{d}^{-1}$, $25\ \text{mM}^{-1}$ NaCl increment: Wyn Jones & Storey, 1978b), the proportional increase in proline is greater, although the former is still quantitatively more significant. In conditions of salt or osmotic shock such as employed by Hanson and Nelson (1978) and in some experiments by Wyn Jones and Storey (1978a), the proportional increase in proline content is even greater, so that final proline levels exceed those of glycinebetaine. Thus, in barley, there appears to be a relationship between the relative rates of proline and glycinebetaine accumulation and the method of stress application (see also Coughlan & Wyn Jones, 1980).

It is probable that there are differences in the rates of induction of proline and glycinebetaine in that glycinebetaine increases have only been recorded after 24 h whereas proline accumulation has been recorded in tens of minutes (Singh et al., 1973). However, this is not borne out in the data of Hanson and Nelson (1978), and further experimentation is required.

A major difference between the two solutes lies in their rates of degradation. The data of Bowman and Rohringer (1970) on wheat, Storey (1976) on maize, and Hanson and Nelson (1978) on barley indicate that the rate of glycinebetaine degradation in plant tissue is slow in contrast to proline (Singh et al., 1973). A direct comparison is shown in Figure 4 in an experiment in which both NaCl and polyethyleneglycol stress were applied and then withdrawn. While proline levels decreased rapidly after stress withdrawal, particularly in polyethyleneglycol-treated tissues, glycinebetaine remained almost constant. The relative inertness of glycinebetaine led Hanson and Nelson (1978) to suggest that it might be employed as a cumulative index of water stress in barley.

The differences between the patterns of glycinebetaine and proline accumulation are quite pronounced in the salt tolerant chenopods, *Atriplex spongiosa* and *Suaeda monoica* (Storey & Wyn Jones, 1978b). In these species, glycinebetaine increases, particularly on a dry weight basis at low salinities, which stimulate both the growth of the plants and their succulence. Proline accumulation occurs either as a transient response to the initial salt shock of low salinities or as a long-term effect in plants exposed to very high, inhibitory salt levels. Thus, glycinebetaine is strongly correlated with sap osmotic pressure (*A. spongiosa*, $R = 0.95$, $p < 0.01$; *S. monoica*, $r = 0.98$; $p < 0.01$), whereas proline accumulation mirrors the decrease in yield and fall in tissue water content at high salinities. In barley, even fairly low salinities cause some growth inhibition and a decline in fresh weight to dry weight ratio (Storey & Wyn Jones, 1978a). Thus, the differences between the behaviour of glycinebetaine and proline in these species may be due to their different growth and morphological responses to salt (NaCl), and the absence of a significant succulence and growth response in barley.

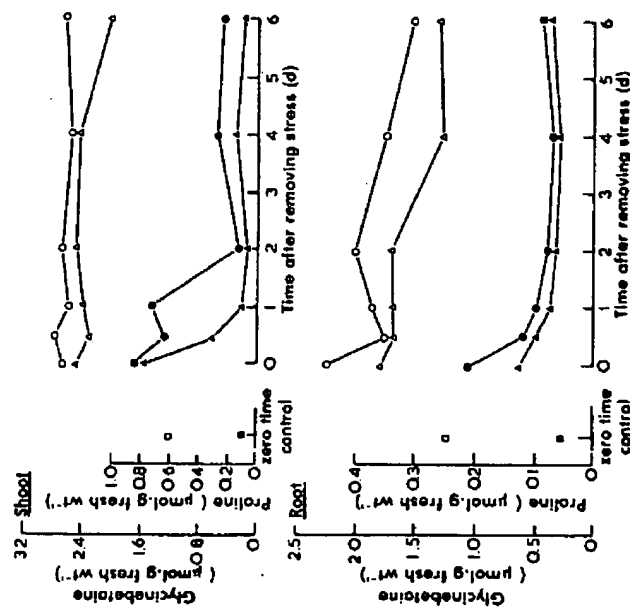


FIG. 4. Comparison of glycinebetaine and proline levels in barley cv. Admar subject to, and then released from, NaCl and water (polyethylene glycol) stress. Open symbols, glycinebetaine (\square , Δ , \circ) subjected to salt (\circ) and polyethylene glycol (Δ) stress. Closed symbols, proline (\blacksquare , \blacktriangle , \bullet , \blacklozenge), subjected to salt (\bullet) and polyethylene glycol (\blacktriangle) stress. The final stress, -0.8 MPa, was applied incrementally over 36 h and maintained for 24 h before being removed at day 0. (Admar and Wyn Jones, unpublished data.)

C. β -ALANINEBETAINES AND PROLINEBETAINES

In *Limnium vulgare*, Larher (1976) found that the shoot levels of β -alaninebetaine increase with external salinity (Table V) to a final concentration (over $100 \mu\text{mol} \cdot \text{g dry wt}^{-1}$), fairly similar to the glycinebetaine levels (highest recorded levels over $400 \mu\text{mol} \cdot \text{g dry wt}^{-1}$). In this species, also, there is a greater proportional increase in proline content although, at the highest salinity, β -alaninebetaine is still, quantitatively, the more important (Table V). Unfortunately, no growth or inorganic ion data were included so

9. Betaines

199

that detailed comparisons with the members of the Chenopodiaceae are precluded.

Whilst the presence of prolinebetaine in alfalfa has been known for many years (Steenbock, 1918), only recently (Wyn Jones & Owen, unpublished data) has it been demonstrated that this compound also accumulates under saline conditions (Table VI). In this work, two alfalfa cultivars of very different salt tolerances were compared, and both the basal and the induced levels of prolinebetaine were found to be much higher in the more tolerant cultivar.

TABLE V
Changes in some soluble nitrogen-containing compounds in young *Limnium vulgare* plants grown at different salinities.^a

Compounds	Control medium ^b	167 mM NaCl ($\mu\text{mol} \cdot \text{g dry wt}^{-1}$)	333 mM NaCl
proline	trace	10.7	65.4
α -alanine	17.7	20.0	19.3
glutamate	15.7	7.7	7.7
amides	72.9	24.7	18.7
γ -aminobutyrate	19.0	11.8	10.8
total free amino acids and amides	147	102	141
β -alaninebetaine	46.9	67.1	100.2

^aData from Larher (1976).

^bPlants grown in Hargreaves' medium with the addition of NaCl as indicated.

TABLE VI
Effect of salinity on the levels of prolinebetaine and related compounds in the shoots of alfalfa cv. 'Hawassi' (2nd cut).^a

Salinity level ^b (mM NaCl)	Sap osmolality (m osmol)	Proline- betaine	Proline	Choline	Trigonelline compounds	Total onium
0	590	12	3.6	1.6	>0.5	18
50	625	15	1.2	2.6	>0.5	18
100	813	17	2.3	2.3	>0.5	19
200	930	18	4.1	0.5	>0.5	23

^aUnpublished data from Wyn Jones and Owen.

^bPlants grown in Hargreaves' medium with added NaCl as shown.

VI. POSSIBLE PHYSIOLOGICAL FUNCTIONS

A. GLYCINEBETAINES AS A CYTOPLASMIC OSMOTICUM

Early studies (Cromwell & Rennie, 1954a, 1954b; Byerum et al., 1955) suggested that glycinebetaine might be involved in methyl transfer, but in some cases (Srinney & Kirkwood, 1954) the glycinebetaine methyl group was not transferred readily to a range of acceptors. This work failed to explain the high concentrations found in some plants. However, the close correlation between glycinebetaine concentration and sap osmotic pressure in leaves of various species (Wyn Jones et al., 1977a, 1977b; Storey & Wyn Jones 1978b, 1979) indicated that the compound might have an osmotic role. Evidence of the sensitivity of some cytoplasmic enzymes and organelles from halophytic plants to high concentrations of inorganic ions (Flowers et al., 1977), and increasing evidence of ion compartmentation between cytoplasm and vacuole (see Jeschke, 1979; Wyn Jones et al., 1979), led a number of workers (e.g. Flowers, 1975; Osmond, 1976) to postulate a requirement for cytoplasmic non-toxic osmolytes. Wyn Jones and his colleagues (1977a, 1977b) produced evidence that glycinebetaine might fulfil this role in some plants. They also suggested that the cytoplasm would be K^+ -specific, with the majority of the Na^+ and Cl^- ions, if present, sequestered in the vacuole. Recently the possible free ion concentrations (activities) in the cytoplasm have been considered in some detail (Wyn Jones et al., 1979), and a unifying hypothesis involving the effects of ions on protein synthesis has been advanced.

If glycinebetaine is utilized as a cytoplasmic osmoticum when the cell sap osmotic pressure exceeds a basal level (about 0.8–1.0 MPa) (Wyn Jones et al., 1977a), then two basic requirements must be met: (i) the solute must be non-toxic to cytoplasmic functions; and (ii) the solute must be preferentially located in the cytoplasm as the concentrations observed can only make a major osmotic contribution if the solute is restricted to a fairly small percentage of the cell volume. (This requirement may also be related to the evidence for the preferential localization of Na^+ and Cl^- in the vacuole: see Jeschke, 1979.)

It may also be suggested that the cytoplasmic osmoticum must be highly water soluble and not carry a net charge which could affect the charge balance of the cytoplasm. Glycinebetaine is dipolar (zwitterionic) and, unlike amino acids, has only one titratable group, the carboxyl, which has a pK_a of 1.83 at 25°C, as the cationic charge on the nitrogen is fixed. Thus, over the physiological pH range, glycinebetaine will have no net charge and no buffering capacity. The internal charge compensation is such that it will pass through a mixed bed ion exchange column at neutral pHs (Caruthers et al., 1960). Glycinebetaine is extremely water soluble (157 g/100 ml⁻¹ at 19°C), but, effectively, is insoluble in ether (Dawson et al., 1969).

9. Betaines

201

Evidence that glycinebetaine, in concentrations up to $0.5\text{--}1.0 \times 10^3$ mM, did not inhibit malate dehydrogenase (decarboxylating) was presented by Wyn Jones et al. (1977b) and confirmed by Flowers et al. (1978). A more detailed study (Pollard & Wyn Jones, 1979) has shown that a number of plant and animal enzymes are not inhibited by glycinebetaine and that the $NaCl$ inhibition of some enzymes is partially relieved by glycinebetaine (200–500 mM). The partial protection probably is not mediated by direct binding of glycinebetaine to the enzyme (cf. Schobert, 1977). We have also found that active intact mitochondria from wheat embryo (Pollard & Wyn Jones, unpublished data), and Type A chloroplasts from spinach leaves (Larkum & Wyn Jones, 1979) may be isolated and studied in glycinebetaine as an alternative osmoticum to sucrose, mannitol or sorbitol. Polyome stability is unimpaired by 500 mM concentrations (Brady & Wyn Jones, unpublished data), while *in vitro* protein synthesis using the wheat embryo system is not significantly inhibited by 250–300 mM glycinebetaine over and above a basal 120 mM potassium acetate medium (Speirs, Brady & Wyn Jones, unpublished data). Thus, the non-toxicity of glycinebetaine is firmly established and the first condition for its role as a cytoplasmic osmoticum has been satisfied.

The second criterion, namely conclusive evidence for a preferential, but not necessarily exclusive, cytoplasmic localization, is more difficult to demonstrate. Nevertheless, three lines of evidence point in this direction.

1. Analyses of tissues of low vacuolation, for example *Atriplex spargansia* apices, wheat embryo or alumnine, showed very high glycinebetaine concentrations, whereas vacuolated, older tissues have lower levels (see Section III).
2. By a histochemical technique, Hall et al. (1978) showed glycinebetaine to be exclusively located in the cytoplasm of *Suaeda maritima* leaf cells.
3. Employing the technique of whole vacuole isolation developed by Leigh and Branton (1976) using red beet storage tissue, Leigh, Ahmad and Wyn Jones (unpublished data; see also Wyn Jones et al., 1977a) found glycinebetaine to be partially excluded from vacuoles.

All the individual techniques may be criticized, but taken together, the results support the original hypothesis and make a strong case for glycinebetaine being largely cytoplasmic. This hypothesis may be applied to halophytes and certain xerophytes, but it is not relevant to ephemeral or succulent xerophytes which maintain low sap osmotic pressure (Walter, 1971). In one sense the concept is more applicable to salt-accumulating halophytes. This is because glycinebetaine, in contrast to proline, is very slowly metabolized in plants and is a semi-permanent end product of metabolism. There is evidence that Na^+ is also held relatively immobile in vacuoles of some plants and is not transported rapidly out of leaves (Yeo,

1976; Jeschke, 1979), so that the accumulation of the metabolically inert glycinebetaine in the cytoplasm may be considered as balancing the absorption of rather immobile Na^+ salts into the vacuoles. In this context, there is unfortunately a lack of good evidence on the distribution of Cl^- (Wyn Jones et al., 1979), but it may also be vacuolar. In the Chenopodiaceae, where this model may be applied best, the major balancing vacuolar anion may be oxalate (Osmond, 1968). Many of these Chenopodiaceae are xerohalophytes and are found in dry, saline habitats. There is a clear relationship in many cases between salt and water stress tolerance.

B. OTHER ASPECTS OF THE PHYSIOLOGICAL CHEMISTRY OF GLYCINEBETAINES

Glycinebetaine has been presented as a solute notable for its lack of toxicity and intracellular metabolic stability. It is, however, rapidly degraded extracellularly by soil micro-organisms (Kortstee, 1970; Wyn Jones et al., 1973). Early work on the addition of glycinebetaine to root media was hampered by microbial interference, but, by using axenic cultures, the positive effects of this compound were demonstrated. Changes in the shoot K^+ and Na^+ contents of treated plants have also been observed (Storey, 1976; Ahmad, 1978). Recently, the possible influence of external glycinebetaine and glycinebetaine loading of tissues on ion fluxes has been studied in greater detail (Ahmad, 1978; Ahmad & Wyn Jones, 1978). Perhaps the most interesting of the phenomena observed was the influence of glycinebetaine loading of barley roots on tonoplast fluxes (Ahmad, Jeschke & Wyn Jones, unpublished data). Using a technique which allowed the separate determination of ^{22}Na efflux and transport in excised barley roots, glycinebetaine loading, or a metabolic consequence thereof, was found to increase Na^+ flux from the cytoplasm to the vacuole and to increase the vacuolar Na^+ concentration. This observation implies a link between the accumulation of the cytoplasmic osmoticum and the partitioning of Na^+ between cytoplasm and vacuole (Table VII). It further suggests, not surprisingly, that the passive model of cytoplasmic osmotic regulation may be superseded by a dynamic model, as various feed-back controls are clearly required between cytoplasm and vacuole to maintain a uniform water potential across the tonoplast. One should also note the structural similarity of glycinebetaine and other *onium* compounds found in nature to compounds well known to modify membrane behaviour (Fluck & Jaffe, 1974; Davidson, 1976).

It is not difficult to relate suggestions that glycinebetaine may have a role in frost resistance (Bokarev & Ivanova, 1971; Sakai & Yoshida, 1968) to the evidence presented above. Similarly, Rafaeli-Eshkol (1968) and Rafaeli-Eshkol and Avi-Dor (1968) originally reported that choline, probably after

9. Betaines

203

TABLE VII

Influence of glycinebetaine loading of excised barley roots on ^{22}Na fluxes. Roots loaded in presence of 1.0 mM glycinebetaine for 24 h.^a

Tissue	Plasmalemma Influx	Efflux $\mu\text{mol. g fresh wt}^{-1} \text{h}^{-1}$	Tonoplast influx	Vacuole content $\mu\text{mol. g fresh wt}^{-1}$
Glycinebetaine not loaded	0.65	0.41	0.047	23.0
loaded	0.70	0.47	0.082	30.1

^aData from Ahmad, Jeschke and Wyn Jones (unpublished).

conversion to glycinebetaine, increased the salt tolerance of a moderately tolerant bacteria *Ba*. More recently, Shkedy-Vinkler and Avi-Dor (1975) have shown the importance of glycinebetaine in protecting a membrane function (respiration) in bacteria exposed to NaCl . They did not stress an osmotic role for glycinebetaine but their data showed a salt-promoted uptake of the solute and an intracellular glycinebetaine concentration of 800 mM under the high salt condition. Thus, it is possible that the solute has an osmotic role as well as a membrane modifying function. These two roles are not necessarily incompatible.

C. POSSIBLE FUNCTION OF β -ALANINEBETAINES

Little is known of the metabolic function of β -alaninebetaine. While the leaf content of β -alaninebetaine increased with external salinity (Larher, 1976), interpretation is complicated by a significant, but ill-defined, proportion being present as the choline ester. As a choline ester this compound, and the 2-trimethylamino-6-ketoheptanoate, would have two fixed cationic charges and would require balancing anions. In contrast to the evidence for glycinebetaine, Larher (1976) observed, by a cytochemical technique, that β -alaninebetaine and/or its choline ester, were sited in the vacuole. If this is confirmed, the rather lower levels found would exclude any simple osmotic role. Drawing an analogy with the structures of acetylcholine antagonists, Larher tentatively suggested that the β -alaninebetaine choline ester may have a role in controlling the permeability of membranes to Na^+ and Cl^- . Although Larher's work did not include inorganic ion analyses, Mg^{2+} might be added to these ions as other data show the Plumbaginaceae to be very rich in this ion (Storey et al., 1977). A further factor worth considering in future work may be a similarity of these compounds to the polyamines, for example spermidine (Basso & Smith, 1974).

VII. CONCLUDING DISCUSSION

A compound which accumulates in a plant under conditions of water or salt stress may be a by-product, possibly toxic, of that stress, or a possible adaptation to help withstand the effects of the stress. Hanson and Nelson (1978) take the view that the only interpretation of glycinebetaine accumulation justified by the data is the former, and they see both glycinebetaine and proline accumulation as a method for consuming excess reducing power. However, from a consideration of its taxonomic distribution and the glycinebetaine responses of salt tolerant members of the Chenopodiaceae, we take the view that glycinebetaine accumulation is an adaptive response to salt and, possibly, water stress. This view is reinforced by an examination of glycinebetaine distribution in other species, such as marine invertebrates, where the compound appears to be accumulated as an intracellular osmoticum. Further, circumstantial support comes from the comparative data on the effects of inorganic ions and glycinebetaine on enzymes, ribosomes, mitochondria and chloroplasts, which show that glycinebetaine, but not the inorganic ions, is tolerated at high concentrations.

It does not follow from this analysis that the rate or degree of glycinebetaine accumulation is a measure of tolerance (see Wyn Jones & Storey, 1978b). Many physiological and biochemical factors are involved in salt and drought tolerance, and the ability to accumulate glycinebetaine, albeit as an important cytoplasmic osmoticum, may not be limiting. While this solute appears to be the preferred osmoticum in some species, other species have evolved other mechanisms and, in all probability, other cytoplasmic osmotica, of which proline may be an example. There is therefore, *a priori*, no conflict between the hypotheses regarding the two solutes. Nevertheless, some differences exist between the two, particularly in their rapidity of accumulation and metabolic lability, which suggest that proline accumulation could be the major response to a transient stress or shock, and glycinebetaine to a longer, possibly more gradual, stress usually involving net salt accumulation.

We have suggested that the concept of a cytoplasmic non-toxic solute is applicable to seeds and other partially dehydrated tissues such as anthers, as well as xerophytic and halophytic plants. These, indeed, may be examples in which the accumulation of the more metabolically inert solute is favoured. It will be apparent from Section II that a wide range of betaines have been found in seeds. Whether they all fulfil an osmotic-protective role similar to that proposed for glycinebetaine remains to be seen, but their structures and the evidence on the role of β -alanine choline ester makes such a simple explanation unlikely.

CHAPTER 10

Proline Accumulation: Physiological Aspects

D. ASPINALL AND L. G. PALEO

I.	The response.....	206
A.	Distribution of the response among living organisms.....	206
B.	Control of accumulation by water potential.....	206
C.	Variation in proline concentration with time.....	209
D.	Distribution of accumulated proline.....	209
E.	Proline loss with relief of water deficit.....	211
F.	The role of plant hormones.....	211
G.	Accumulation in response to other environmental stress factors.....	213
	Consequences of proline accumulation.....	214
A.	Functional aspects of proline accumulation.....	214
B.	Osmoregulation.....	215
C.	Hydration characteristics of biopolymers.....	215
D.	The conservation of energy and amino groups.....	216
E.	Sink for soluble nitrogen.....	217
F.	Senescence phenomena.....	217
	Proline accumulation and stress resistance.....	219
A.	General considerations.....	219
B.	Hardening.....	219
C.	Growth retardants.....	220
D.	Exogenous proline application.....	221
E.	Comparisons between species.....	222
F.	Comparisons within species.....	230
	Selection for proline accumulation.....	237
A.	Hybrid selection.....	237
B.	Selection within a cultivar.....	239
	Future prospects.....	240
II.		
III.		
IV.		
V.		

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Drought and salt tolerance: towards understanding and application

Kent F. McCue and Andrew D. Hanson

Only a few desirable traits in plants are understood at the molecular level. Fewer still are amenable to manipulation by molecular biological techniques currently available. The accumulation of glycine betaine, an osmoprotectant compound implicated in drought and salt tolerance, may be one such trait.

By far the most important factors limiting crop productivity are environmental stresses, of which lack of water (drought) is the most serious¹. Salinity is also a major constraint; this is a significant problem in the USA because it affects some of the otherwise most productive agricultural areas, including almost 30% of California's irrigated land². Increased drought- and salt-tolerance have therefore been major objectives of plant breeding programs for regions where rainfall or irrigation supply is limiting, where soil salinity is high, or where water quality is poor. Although conventional breeding methods have had some encouraging successes, overall progress has been slow¹. This has focused interest on the possi-

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bilities of genetic engineering for drought- and salt-tolerance³. Is this interest justified? We think it is, provided that it is tempered by an understanding of the inherent limitations of the genetic-engineering approach.

Adaptations of plants to stress

Plants have evolved many types of adaptations to drought and salinity which can be classified as belonging to one of four levels. The most complex mechanisms, requiring the interplay of many gene products, are at the developmental level; the simplest, perhaps involving only a single gene product, at the biochemical level (Fig. 1). Unfortunately, with the current levels of knowledge of plant genetics and metabolism, genetic engineering can only be applied to biochemically definable traits; desirable but complex traits remain out of reach. Such complex traits include water-use efficiency (the amount of dry matter or harvestable yield produced per

unit of water used)⁴, root morphology and anatomy⁵, and the ability to exclude salt while maintaining water flow through the plant⁶. While biochemical adaptations do lie within reach of genetic engineering, our understanding of integrative processes in plants is not yet adequate for predicting whether alteration of these traits will translate into beneficial effects at the crop level.

Osmoprotectants in plants

What kinds of biochemical stress-resistance traits are sufficiently defined for genetic engineering? Here we present the case in favor of glycine betaine accumulation, beginning with some background on the physiology of drought and salt stress. A problem for all living organisms in dry or saline environments is to maintain water content; this is achieved by solute accumulation, which lowers solute potential. The solutes accumulated in the cytoplasm must be non-toxic ('compatible') with respect to metabolic processes; that is, they should not interfere with protein structure or function when present at high concentrations⁷. Only a few types of organic compounds (osmoprotectants) meet this requirement; quaternary ammonium compounds such as glycine betaine are among the commonest of these, and occur in bacteria, cyanobacteria, algae, higher plants and animals⁸. Other osmoprotectants found in diverse organisms include various polyols, the amino acid proline, and tertiary sulfonium compounds. Many other solutes, especially inorganic ions such as Na⁺, Cl⁻

and SO_4^{2-} , are quite toxic. For salinity stress, where the cytoplasm may be exposed to elevated levels of toxic ions, protection of protein function in the presence of such ions ('haloprotection') is desirable. Studies with cyanobacteria have shown that of several compatible solutes examined, only glycine betaine provided haloprotection, relieving the inhibitory effects of high salt levels on enzyme activity⁹.

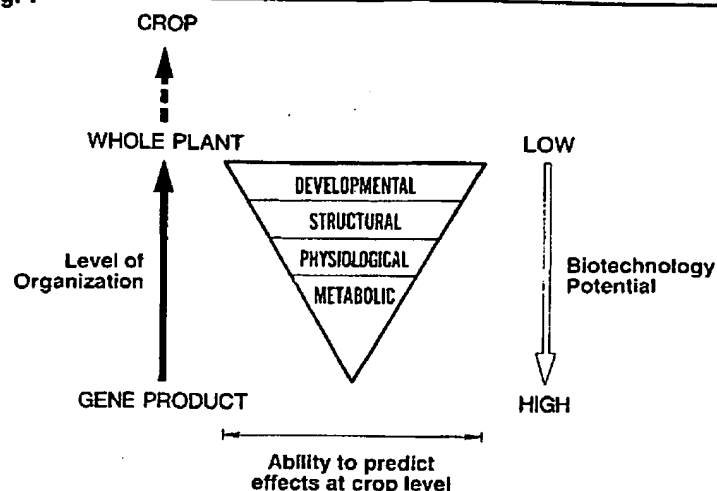
Figure 2 shows the chemical structures of glycine betaine and some other osmoprotectants found to accumulate in plants, and the legend gives information on their taxonomic distribution. Glycine betaine accumulation is quite widespread, occurring in species from many large plant families such as the Asteraceae, Chenopodiaceae, Poaceae and Solanaceae¹⁰. Interestingly, glycine betaine does not accumulate in many important crop species such as tomato, potato and rice, all of which have relatives which are accumulators. This makes such species good targets for genetic engineering of the glycine betaine pathway. Some of the less common osmoprotectants shown in Fig. 2 may also become of interest for genetic engineering as more is learned about them.

A very important point about the accumulation of glycine betaine (and probably other osmoprotectants) in higher plants is that this accumulation is restricted largely to the cytoplasmic compartments of the cells (Fig. 3a). The compartmentation of glycine betaine depends, presumably, upon specific transport mechanisms, but very little is known about betaine transport systems in plants, and it is not clear whether or not such transport systems are specific to betaine-accumulating species.

Glycine betaine biosynthesis

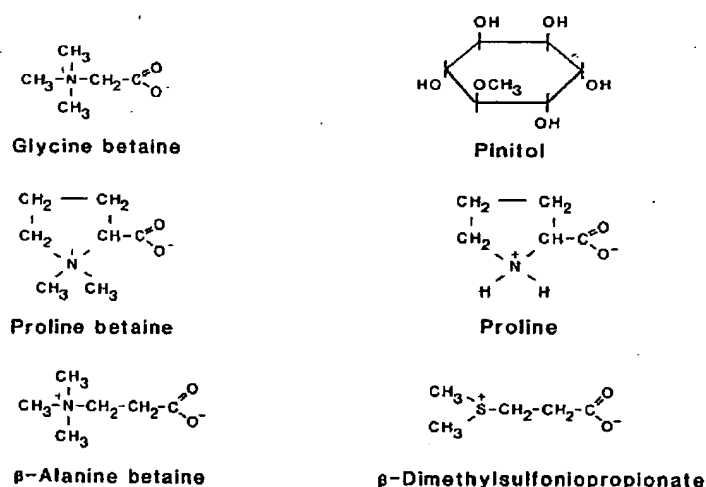
The synthesis of glycine betaine requires only two enzymes in a two-step oxidation of choline (Fig. 4). The first enzyme, choline mono-oxygenase (CMO), has been partially purified and characterized from spinach²⁴. CMO is localized in the chloroplast stroma; it is stimulated by Mg^{2+} and has a pH optimum of ~8. The reaction catalysed by CMO requires molecular oxygen and reduced ferredoxin²⁴. The second enzyme is betaine aldehyde dehydrogenase (BADH). The majority

Fig. 1



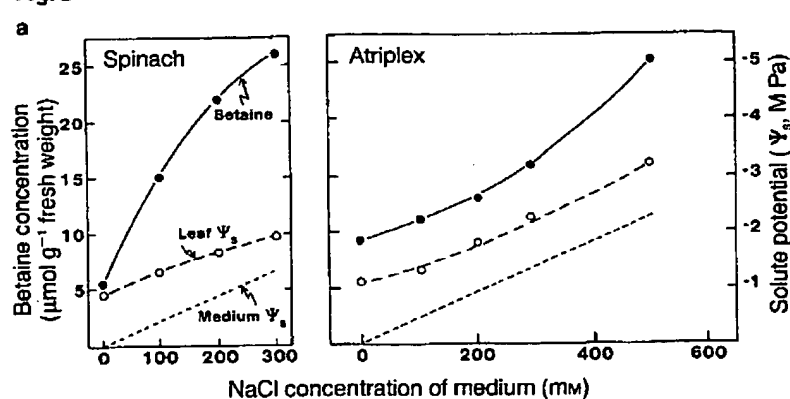
Some relationships between stress resistance and biotechnology. Crop stress resistance can be viewed as the sum of traits expressed at four levels of organization. At the highest level are developmental traits (e.g. time of flowering). Below this come structural traits (e.g. rooting patterns or leaf waxiness). The next lower level comprises physiological traits such as water-use efficiency. At the lowest organizational level are metabolic or biochemical resistance traits; since these traits can be construed in terms of small numbers of enzymes (gene products), they are potential targets for biotechnology, though it is difficult to predict the impact on crop yield of intervening at the biochemical level. The organizational levels are framed by a triangle whose width is proportional to our ability to predict the effects of genetic manipulation on stress resistance at whole-plant and crop levels; for example, intervention at the developmental level resulting in an earlier/late time of flowering will predictably alter stress resistance, whereas the effects on resistance of altering metabolic traits, such as betaine accumulation, are harder to predict.

Fig. 2



Some compatible solutes found to accumulate in higher plants. Certain of these occur very widely, such as proline. Glycine betaine accumulation occurs in several large and important families including the Asteraceae, Chenopodiaceae and Poaceae. Other betaines have a more limited distribution, such as proline betaine found in the Fabaceae and the Lamiaceae, and β -alanine betaine in the Plumbaginaceae. Other compatible solutes are also limited in occurrence, such as o-pinitol found primarily in the Fabaceae and some Caryophyllaceae, and β -dimethylsulfoniopropionate, found in the marine grass *Spartina*. (Data from Refs 10–13.)

Fig. 3



b Solutes in a salinized chenopod leaf cell

	K ⁺	Na ⁺	Cl ⁻	Organic acids	Betaine
	(mm)				
Vacuole	150	200	150	100	< 1
Cytosol and chloroplast	120	< 50	< 50	60	300

Glycine betaine as a compatible cytoplasmic solute in plant cells. (a) Typical patterns of osmotic adjustment and betaine accumulation in leaves of salt-stressed plants of the family Chenopodiaceae. Spinach is representative of moderately salt-tolerant plants of this family, whereas Atriplex spongiosa is a more halophytic species. In both species, as salt is added to the growth medium, the leaves accumulate betaine and other solutes, maintaining their solute potential below that of the medium by a fairly constant 0.6 to 1 MPa. This differential provides the driving force for growth and water uptake. (Data from Refs 14, 15.) (b) The major solutes which accumulate in salinized leaves are Na⁺, Cl⁻, organic acids, and glycine betaine. Salinization causes relatively little change in vacuolar and cytoplasmic K⁺ levels. Na⁺ and Cl⁻ accumulate mainly in the vacuole, and betaine mainly in the cytoplasm (chloroplasts + cytosol). The vacuole occupies about 90% of the cell volume, and the chloroplast and cytosol about 5% each. Hence osmotic adjustment of the bulk of the cell water is achieved with Na⁺ and Cl⁻, which are readily available from the salinized growing medium, but are toxic to metabolism. Glycine betaine, which is non-toxic but energetically expensive, is used for osmotic adjustment only in the crucial metabolic compartments. (Data from Refs 16–21.)

of BADH activity is in the chloroplast stroma, although there may also be a minor cytosolic isoform. BADH has a preference for NAD⁺, and has a similar pH optimum to that of CMO²⁵. We have recently cloned cDNAs for BADH from spinach²⁶, and are currently working on the cDNA cloning of CMO.

The activities of the glycine betaine synthetic pathway enzymes are regulated by osmotic stress. CMO activity is increased threefold by treatment of spinach plants with 200 mM NaCl²⁴. Likewise, BADH protein and mRNA levels are increased several-fold by salt treatment^{26,27}. At present, it is not known whether control of BADH mRNA amounts is at the transcriptional, or post-transcriptional level, or both.

Choline, the substrate for CMO, is ubiquitous in nature and its biosynthesis is under feedback control in all plants tested^{28–30}. A priori, it therefore seems likely that the supply of choline would be adequate to support the additional demand caused by introduction of the glycine betaine biosynthetic pathway. However, this assumption should be treated with caution; species appear to differ in the involvement of soluble versus phospholipid-bound intermediates in choline synthesis, perhaps indicating differences in compartmentation of the pathway or its end product^{31,32}.

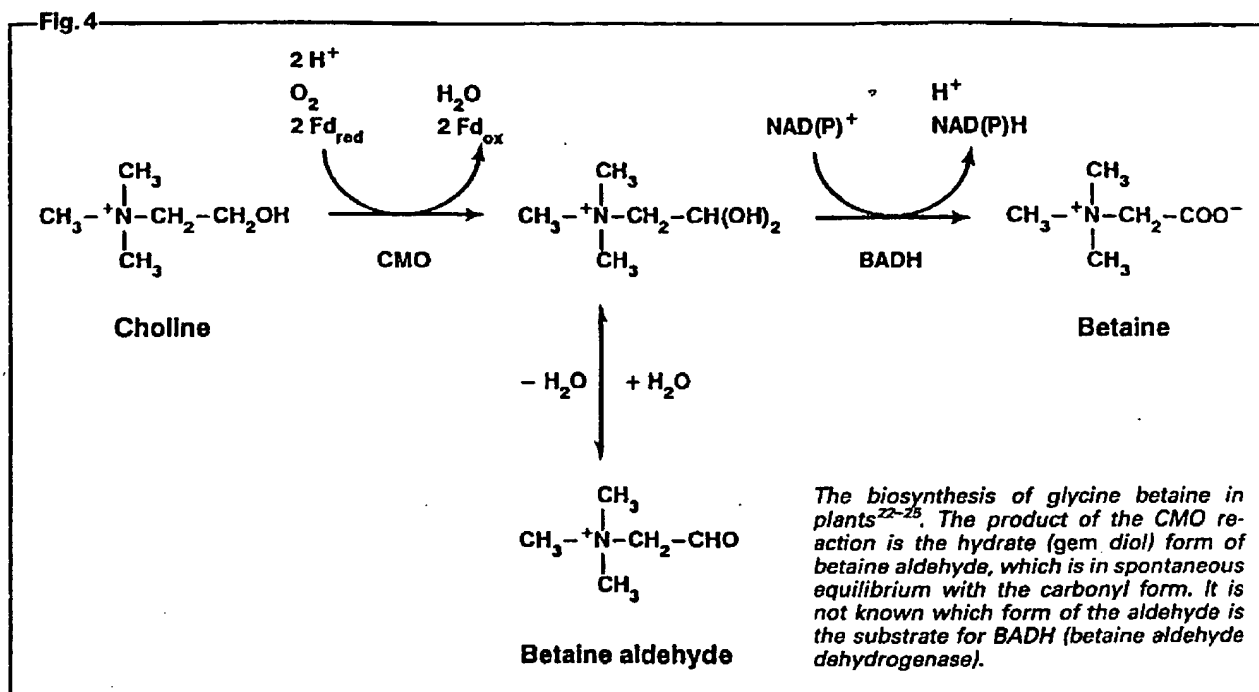
Towards engineering of target species

Glycine betaine accumulation

does not occur in many important crop species into which genes can be inserted with current technology. Some of these betaine-deficient species, such as rice, tomato and potato, are probably the most promising targets for genetic engineering; the fact that these have betaine-accumulating relatives makes it more probable that enough choline will be available in the chloroplast to sustain high rates of betaine synthesis, and that the transport mechanisms required to maintain proper compartmentation of betaine will be present.

What might be the effect of introducing the betaine pathway into a non-accumulator on productivity under stress? As yet, this cannot be answered directly. However, comparing biomass yields of wild-type, betaine-accumulating maize genotypes with those carrying a naturally occurring betaine-deficiency mutation showed a yield advantage of about 12% for the betaine-accumulators at a dry site in Mexico (C. Lerma, J. Bolaños, D. Rhodes and A. D. Hanson, unpublished). An effect of this magnitude on production is the most that can reasonably be expected from introducing a single trait³³.

Having selected a target crop species, a number of assumptions must be made in order to proceed (Fig. 5). For example: glycine betaine is osmoprotective in a wide variety of organisms, so it is likely to function similarly in plants that do not naturally accumulate it, though this will need to be proved for each target species. The biosynthesis of glycine betaine from choline is fairly well understood and as genes are isolated for both steps in the pathway, we may assume that they will include the sequences necessary for targeting to the chloroplast. The production of the necessary levels of active enzyme product should be attainable using constitutive promoters already available, although these will probably not reproduce the stress-inducibility which characterizes the natural betaine pathway. Whether correct localization of glycine betaine in the cytosol and chloroplast will occur is not predictable at this point. We do know that non-accumulators such as tobacco show active uptake of supplied glycine betaine³⁴, although the subcellular



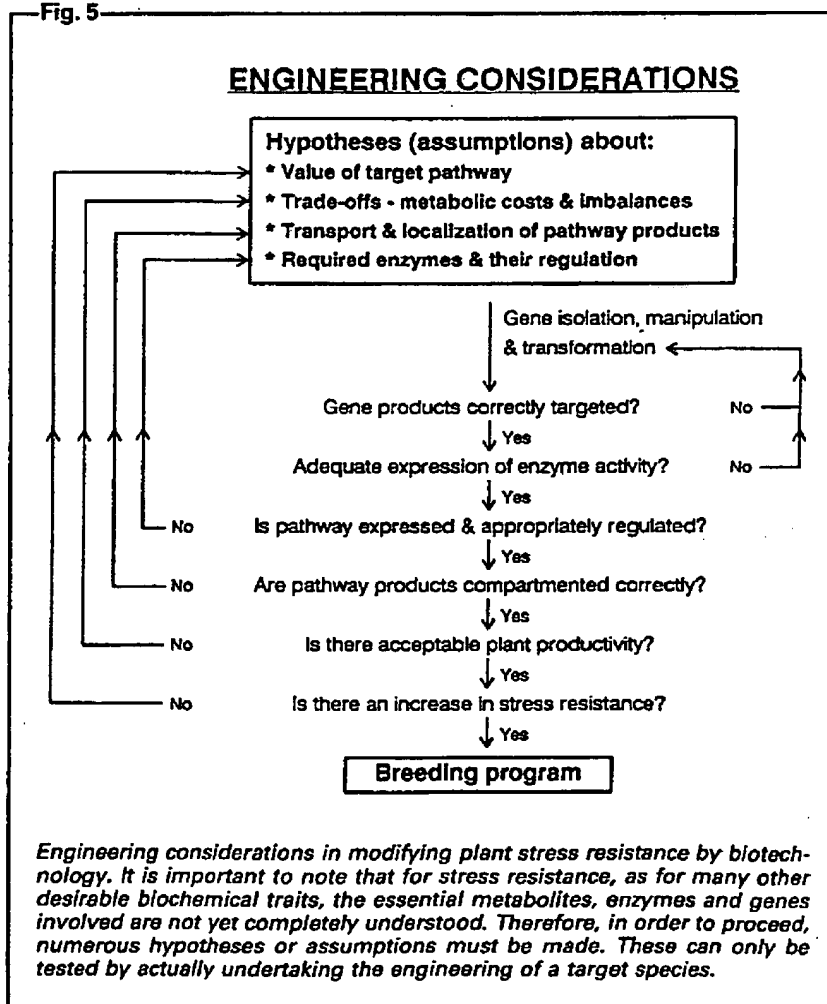
location of the absorbed betaine has not been determined.

Supposing the preceding assumptions hold, then transgenic plants which accumulate betaine in the appropriate compartments will be obtained. Field performance of these transgenic plants will then have to be compared with controls, in the absence and presence of stress. Suppose that performance (yield) under stress is improved, but is poorer in the absence of stress, and that betaine accumulation proves not to be stress-regulated in the engineered plants. Such an outcome would indicate the desirability of a more ambitious engineering project – the engineering of stress-inducibility, which could be achieved by placing the genes of the pathway under the control of an osmotically responsive promoter. Isolation of such a promoter is possible (but has not yet been done), but its proper functioning would probably require *trans*-acting factors as well as signal detection and transducing machinery to be present in the target plant. If the elements of such a network are not present, their identification and isolation would demand much additional basic research.

Possible engineering problems

We have indicated several ways in which engineering glycine betaine accumulation into transgenic plants may not be straightforward, and that

Fig. 5



the effect on stress resistance is not predictable. Such uncertainties can only be confirmed or dismissed by carrying out some pioneering engineering experiments (Fig. 5). Until the genes for CMO and BADH are introduced and the transgenic plants evaluated, we cannot be certain that these genes are sufficient for betaine synthesis and accumulation. Correct targeting of the enzymes to the chloroplast is another possible obstacle, especially when transferring genes from monocots to dicots³⁵. The substrate choline must be present in the chloroplast; this may require *in-situ* synthesis or the proper transport mechanisms, and the known differences in the biosynthesis of choline between plants^{31,32} may affect the choline availability. Compartmentation of glycine betaine may depend upon specific transport mechanisms in the chloroplast envelope, tonoplast and plasma membrane, possibly not present in all plants; this can be substantiated only after successful transfer of the biosynthetic pathway.

Successful expression of betaine accumulation might, in itself, create problems. Betaine synthesis could compete detrimentally with choline utilization in phospholipid synthesis, causing an overall poorer performance in the engineered plant. There could also be more general effects resulting from increased carbon and nitrogen partitioning into the betaine synthetic pathway; although calculations suggest that these should be inconsequential^{36,37}, there is no way of being certain in advance.

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Book-Reviews Supplement: Contents

Biotechnology - A Textbook of Industrial Microbiology, 2nd edn (by W. Cruieger and A. Cruieger), by J. M. Woodley	363	Biotechnology Japan (by M. D. Dibner and R. S. White), by A. Scheidegger	367
Presentation of Clinical Data (by B. Spilker and J. Schoenfelder), by R. Mather	363	Cryopreservation of Thin Biological Specimens for Electron Microscopy: Methods and Applications (by N. Roos and A. J. Morgan), by J. Dubochet	368
Biotechnology in the Food Industry (edited by M. P. Tombs), by J. D. R. Hille	364	Applied Biosensors (edited by D. L. Wise), by I. J. Higgins	369
Microbial Water Stress Physiology: Principles and Perspectives (by A. D. Brown), by R. H. Reed	365	Microbial Mineral Recovery (edited by H. L. Ehrlich and C. L. Brierty), by R. K. Poole	370
Applications of Biotechnology in Forestry and Agriculture (edited by V. Dhawan), by B. H. McCown	365	Biotechnology of Biomass Conversion (by M. Wayman and S. R. Parekh), by B. S. Hartley	370
Genetic Engineering and Biotechnology Related Firms, 9th edn: Worldwide Directory, 1990-1991 (edited by M. Sittig); and Biotechnology Guide Japan 1990-1991: Company Directory and Comprehensive Analysis (edited by Nikkei Biotechnology), by M. D. Dibner	368	Methods in Molecular Biology, Vol. 5: Animal Cell Cultures (edited by J. W. Pollard and J. M. Walker), by D. Knight, G. Shah and G. Gough	371

J. Plant Physiol. Vol. 141, pp. 188–194 (1993)

Proline Accumulation as Drought-tolerance Selection Criterion: its Relationship to Membrane Integrity and Chloroplast Ultrastructure in *Nicotiana tabacum* L.

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Summary

The value of proline accumulation as a criterion in selecting for drought-tolerance, was evaluated in four tobacco cultivars of differing drought-tolerance. Proline determination, measurement and calculation of a membrane integrity index (MII), and ultrastructural observations were conducted simultaneously under controlled environmental conditions during the stress period. Water stress of increasing severity ($0.2-0.3 \text{ MPa d}^{-1}$) that ranged from light (-0.52 MPa) to severe (-2.51 MPa) was induced by withholding water. A substantial accumulation of proline was observed in all four cultivars, the extent of which correlated positively with their individual drought-tolerance. Ultrastructural observation indicated that the drought-tolerant cultivars mobilized the, more than adequate, store of starch to a greater extent than the drought-sensitive cultivars during stress. Water stress-induced membrane damage occurred earlier and was much more severe in the drought-sensitive cultivars, as their MII-values already rose to between 20.4% and 23.4% at a Ψ_L of -1.27 MPa , while those of the drought-tolerant cultivars only reached values of 18.5% and 20.9% at a Ψ_L of -1.67 MPa . The Ψ_L at which proline levels start to increase dramatically (the shorter the response time the better) and the end concentrations of proline accumulated, are advocated as criteria to be used in selecting for drought-tolerant tobacco genotypes as early as the F_1 - or F_2 -generations.

Key words: *Nicotiana tabacum* L., membrane integrity, proline accumulation, selection criterion, starch content, ultrastructure, water stress.

Abbreviation: MII = membrane integrity index.

Introduction

Within the leaves of many plants subjected to moderate or severe water stress, one striking change in nitrogen metabolism is the accumulation of free proline as a result of net *de novo* synthesis from glutamic acid (Barnett and Naylor, 1966; Boggess et al., 1976). Water stress-induced proline accumulation can account for as much as 1% of the dry leaf matter in many species; this, however, only takes place if there are adequate carbohydrates present in the tissue (Hsiao, 1973). Proline has been shown to ameliorate the deleterious effects of heat, pH, salt, chemicals and dehydration

on enzyme activity and organelle systems (Ahmad et al., 1979; Bowlus and Somero, 1979; Nash et al., 1982; Yancey and Somero, 1979). The mechanism whereby these «compatible» solutes (Schobert and Tschesche, 1978) exert their influence has, however, not yet been elucidated (Stewart, 1989), nor are the molecular mechanisms underlying these effects completely understood. Proline action has been suggested to involve effects on the hydration layer surrounding phospholipids and possibly also its intercalation between phospholipid head groups (Rudolph et al., 1986). Furthermore, it is striking that many of the solutes which accumulate in stressed plants and which have protective

properties are also reported to reduce free radical activity. In this regard it has been indicated that proline can also detoxify free radicals by forming long-lived adducts with them (Floyd and Zs-Nagy, 1984). Just as little is known about the changes which the tonoplast and plasmalemma undergo during desiccation, although leakage studies indicate that some changes do occur (Bewley and Krochko, 1982). Many of these studies have been reviewed by Simon (1974, 1978). What is known, however, is that when enzymes, structural proteins, macromolecular complexes, etc., are desiccated in their native state, the integrity of the molecules and structures can be retained if some water remains associated with them, which prevents the formation of unfavourable conformations (Todd, 1972) or fragmentation (Darbyshire and Steer, 1973).

As stated above, it has been suggested for some time that the often observed accumulation of proline in plant tissues during water stress is an adaptive response (Handa et al., 1986). It can be argued, however, that the mere correlation between accumulation and development of stress is not enough proof that the substance has any adaptive value in postponing stress or increasing stress-tolerance and that the accumulation occurs because of disturbance of normal nitrogen metabolism with the result that any beneficial effects, if such exist, are merely coincidental (Kramer, 1983).

As it is very difficult to prove that these compounds have an important adaptive value, this study was conducted as an attempt to gain some evidence, albeit indirectly, as to whether the ability to accumulate proline in four tobacco cultivars, can be positively correlated with their drought-tolerance, because of its protective membrane stabilizing effects. To achieve the latter, it was deemed necessary to find answers to the following questions: Firstly, does a positive correlation exist between proline accumulation, membrane integrity and the known difference in drought-tolerance of the four tobacco cultivars? Secondly, do the four cultivars contain enough carbohydrates to allow proline accumulation and does the extent to which starch is mobilized during water stress differ among the four cultivars? Thirdly, how extensive are the water stress-induced ultrastructural changes and do they differ in the four cultivars? Finally, which, if any, aspect(s) of proline accumulation may be useful as selection criteria in selecting for drought-tolerance?

Material and Methods

In sequence of increasing drought-tolerance the four cultivars of *Nicotiana tabacum* L. under investigation, were TL33, CDL28, GS46 and ELSOMA. These cultivars were selected due to their differing performances in the field during water stress periods (Personal communication: Dr. C. J. Steenkamp, Tobacco and Cotton Research Institute, Rustenburg, RSA). Seed was allowed to germinate in soil in pots. The young seedlings developed under glasshouse conditions with optimal water application (watered once daily to field capacity). Before the onset of experimentation the plants were moved to a growth room (photon flux density, 400–600 $\mu\text{mole m}^{-2}\text{s}^{-1}$; relative humidity, 35%) and allowed to acclimatize for a period of 96 hours. During the acclimatization period the plants were watered twice daily to avoid their experiencing any water stress because of the slightly higher growth chamber tem-

peratures. The growth chambers were lit for 13 hours at 25 °C followed by an 11 hour dark period at 16 °C. Experimentation started when the plants were approximately 90 days old and a water stress of increasing intensity (0.2–0.3 MPa d^{-1}) was induced by withholding water. The water stress period lasted for 12 days and ranged from light (–0.52 MPa) to severe (–2.51 MPa). Leaf water potential determinations were done every second day with a Scholander pressure chamber (PMS-instrument, Oregon, USA). Membrane integrity, measured as ion leakage, was carried out as described by Sullivan and Ross (1979) and modified by Blum and Ebercon (1981). Proline concentration was determined by means of a rapid colorimetric method, developed for plant tissue by Bates et al. (1973). All leaf samples were taken at 08:00. The sixth youngest leaf was used in all instances, as this represented a mature, nearly fully expanded leaf, before the onset of senescence.

For ultrastructural investigation samples of 1 mm³ were cut from the centre of the lamina, lateral to the midrib, while immersed in fixative, and these were immediately transferred to fixative (2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4). After evacuation of 15 minutes, fixation was continued for a further 2 hours. The material was postfixed for 1 hour in 0.5% aqueous osmium tetroxide, dehydrated in acetone and infiltrated with resin (Spurr, 1969). For light microscopy semi-thin sections were made, stained with toluidine blue O (Trump et al., 1961) or toluidine blue O and neofuchsin (Botha et al., 1982). For electron microscopy, thin sections were contrasted with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963).

Results and Discussion

The calculated MII-values (Table 1) of the four cultivars clearly indicate that the membranes of the drought-sensitive cultivars 6TL33 and CDL28 were damaged earlier than those of the drought-tolerant cultivars (GS46 and ELSOMA). This is evident from the fact that the MII-values of TL33 and CDL28 already increased to 20.4% and 23.4% respectively at a Ψ_L of –1.27 MPa whilst the MII-values of GS46 and ELSOMA only rose to similar levels i.e. 20.9% and 18.5% respectively at a Ψ_L of –1.67 MPa. Furthermore, at the severe stress level (–2.51 MPa) the water stress-induced membrane damage was found to be approximately 10% more in the drought-sensitive cultivars. The value of the above-mentioned results is only fully realized when seen in conjunction with the statement of Sullivan and Ross (1979). These authors have been concerned with the relationships between electrolyte leakage following a shock treatment and general ability of plants to tolerate stress for several years. According to them, the evidence they obtained indicated that the degree of membrane stability to stress (as evaluated by ion leakage) correlates well with the tolerance of other plant processes to stress, even if the stress is at levels lower than that needed to measure appreciable ion leakage. These include soluble protein and enzyme resistance to denaturation, maintenance of photosynthesis by intact tissue and other responses to stress (Sullivan and Kinbacher, 1967; Sullivan and Ross, 1979; Kinbacher et al., 1967). In the light of our results (Table 1 and Fig. 1) regarding membrane integrity of the test plants, and because it is the rate of water stress-induced membrane injury that is estimated through the measurement of electrolyte leakage from cells (Blum and Ebercon, 1981), it may be said that not only the membrane

Table 1: Water stress-induced proline accumulation and changes in the membrane integrity index of four cultivars of *Nicotiana tabacum* L., which differ with respect to their drought-tolerance.

Cultivar	Ψ_L MPa	Proline conc., mmol kg ⁻¹ dry mass	% of control	MII % of control
TL33	-0.52	0.60±0.01	100.0	0.0±0.0
	-0.77	1.91±0.04	318.5	3.8±0.6
	-1.27	3.40±0.9	566.7	20.4±0.7
	-1.67	3.72±0.4	620.0	22.4±0.5
	-1.97	9.50±0.3	1583.3	30.4±0.4
	-2.32	18.55±0.3	3019.7	38.5±0.8
	-2.51	35.66±0.7	5943.3	51.1±0.9
CDL28	-0.52	0.81±0.02	100.0	0.0±0.0
	-0.77	1.99±0.01	245.7	4.5±0.3
	-1.27	3.30±0.3	407.4	23.4±0.8
	-1.67	5.45±0.4	672.8	28.4±0.9
	-1.97	5.82±0.9	718.5	32.3±0.9
	-2.32	16.26±0.4	2007.4	36.1±0.5
	-2.51	32.22±0.3	3977.7	52.8±0.6
GS46	-0.52	0.17±0.01	100.0	0.0±0.0
	-0.77	1.19±0.04	700.0	6.2±0.1
	-1.27	2.12±0.03	1247.1	7.4±0.8
	-1.67	6.83±0.5	4017.6	18.5±0.7
	-1.97	14.16±0.2	8329.4	29.4±0.2
	-2.32	30.20±0.8	7764.7	40.6±0.7
	-2.51	41.90±0.2	4647.1	42.9±0.4
ELSOMA	-0.52	0.38±0.01	100.0	0.0±0.0
	-0.77	1.59±0.02	418.0	6.8±0.9
	-1.27	2.05±0.9	539.5	8.9±0.5
	-1.67	11.71±0.3	3081.6	20.9±0.3
	-1.97	23.72±0.2	6242.1	26.7±0.6
	-2.32	44.78±0.5	1784.2	32.5±0.4
	-2.51	47.62±0.7	2531.6	41.1±0.5

MII = $1 - [1 - (T_1/T_2) / 1 - (C_1/C_2)] \times 100$ where according to Blum and Ebercon (1981), T and C refer to mean of treatment and controls, respectively, and subscripts 1 and 2 refer to initial and final conductivities, respectively.

systems of the drought-sensitive cultivars, but also their physiological processes in general were damaged earlier. Furthermore, the extent of the water stress-induced damage, as reflected by the steeper slope of the MII-curves (Fig. 1), was much more severe in these cultivars. In keeping with the idea that the water stress-induced membrane damage was much more severe in the drought-sensitive cultivars, it should also be noted that at a Ψ_L of -2.51 MPa, membrane damage in these cultivars was irreversible. The latter statement is supported by the fact that the MII-values represent the percentage of membrane injury due to desiccation (Blum and Ebercon, 1981) and these values of the drought-sensitive cultivars exceeded 50% at a Ψ_L of -2.51 MPa (Table 1).

According to Bewley and Krochko (1982) direct evidence that species specific differences in membrane composition or protein structure contribute to the tolerance of plants is generally lacking. This view is shared by Schwab and Heber (1984) who concluded that drought-tolerance cannot be explained by a particular membrane structure which makes the membrane insensitive to water stress, but must rather, though it may only be in part, be attributed to the composition of the membrane surroundings, with special reference

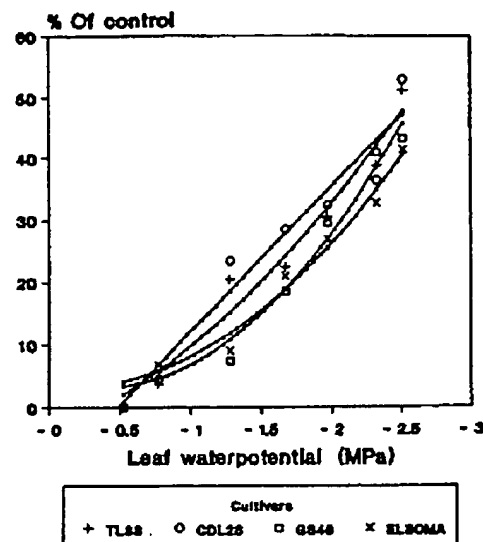


Fig. 1: Effect of water stress on the membrane integrity of four cultivars of *Nicotiana tabacum* L. of differing drought-tolerance.

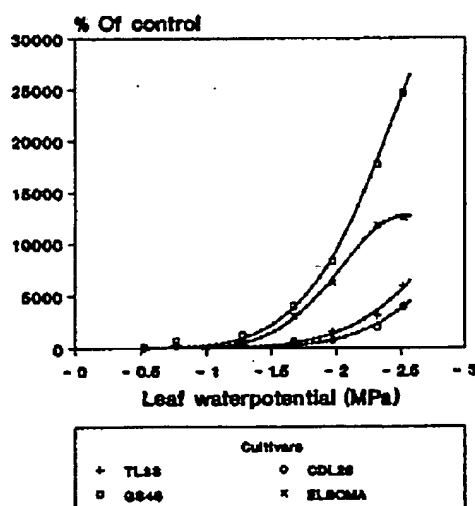


Fig. 2: Water stress-induced proline accumulation in four cultivars of *Nicotiana tabacum* L. which differ with respect to their drought-tolerance.

to membrane compatible solutes. In this regard it is interesting to note that Rudolph et al. (1986) found that in the presence of trichalose, sucrose, proline and glycine betaine, ion leakage is prevented.

In accordance with the results obtained by several authors (Bogges and Stewart, 1980; Handa et al., 1986) in several species, our results showed a dramatic increase in the free proline levels of all four the tobacco cultivars (Fig. 2). At a Ψ_L of -0.77 MPa the proline levels of all four the tobacco cultivars already deviated significantly from the control values. The proline concentrations of the drought-sensitive

cultivars were 1.91 and 1.99 $\mu\text{moles gram}^{-1}$ respectively, which is more than double the proline concentration in the unstressed tissue, while the proline concentration in the drought-tolerant cultivars had already risen to 1.19 and 1.59 $\mu\text{moles gram}^{-1}$, which is four times more than the proline concentration in the unstressed tissue. The base proline levels of the drought-tolerant cultivars proved to be lower than those of the drought-sensitive cultivars (Table 1). Of greater importance, however, is the fact that the drought-

tolerant cultivars were able to accumulate roline earlier and to much higher end concentrations. At a Ψ_L of -1.67 MPa the proline concentrations of both drought-sensitive cultivars were just a little more than six times those of the controls, while that of GS46 was 40 times higher and that of ELSOMA, 30 times higher than their respective controls. At the severe stress level of -2.51 MPa the amount of proline accumulated by ELSOMA was more than three times higher than that of both drought-sensitive cultivars and that of



Fig. 3: Differential water stress-induced ultrastructural changes in four tobacco cultivars of differing drought tolerance: (a) CDL28 ($\Psi_L = -0.52 \text{ MPa}$); (b) ELSOMA ($\Psi_L = -0.52 \text{ MPa}$); (c) CDL28 ($\Psi_L = -1.97 \text{ MPa}$); (d) ELSOMA ($\Psi_L = -1.97 \text{ MPa}$); (e) TL33 ($\Psi_L = -2.51 \text{ MPa}$); (f) ELSOMA ($\Psi_L = -2.51 \text{ MPa}$). Bar = $1 \mu\text{m}$.

GS46 more than four times higher than both the drought-sensitive cultivars (Table 1). With reference to the latter two sets of results, it should be noted that Hsiao (1973) stated that sketchy indications are that the level of proline may be insensitive to mild stress and that accumulation only takes place if there are adequate carbohydrates in the tissue. The accumulated proline apparently came from *de novo* synthesis (Thompson et al., 1966) with glutamate as precursor (Morris et al., 1969) and there is evidence to indicate that carbohydrates are the ultimate source of the skeleton (Stewart et al., 1966).

With regard to carbohydrate reserves of the test plants the following observations relating to starch grains in the chloroplasts were made. Special attention was, however, paid to the state of the chloroplast envelope membranes as well as to that of the inner thylakoids in this investigation, as according to Ferrari-Iliou et al. (1984) chloroplasts can be regarded as the most fragile organelles in the cell. In the unstressed leaves of all four cultivars the presence of large starch grains was striking, although the leaves were sampled after only 2–3 hours of exposure to light (Figs. 3 a and 3 b). The grana were found to be clearly defined, with parallel thylakoids and little dilatation of the intergranal spaces. Osmiophilic globules, some appearing more osmiophilic than others, occurred in most of the chloroplasts studied.

After water had been withheld for 8 days ($\Psi_L = -1.97$ MPa), the number and size of the starch grains of the chloroplasts of leaves of cultivars TL33 and CDL28 (Fig. 3 c), showed little change from the original situation observed in the unstressed leaves. The chloroplast envelope membranes in some cells of cultivar TL33 were not clearly defined, while in others they were still intact. Large lipidic globules were present in some of the chloroplasts. Granal and stromal thylakoids were severely disrupted in some chloroplasts. The chloroplast membranes in leaves of CDL28 were still intact and the position of the plasmalemma with regard to the cell wall showed little change, although signs of shrinking of cytoplasm were discernible in some cells. Few vesicular structures were present in the cells. Stromal thylakoids showed some signs of dilatation in some of the cells, but the grana were still intact. The size of starch grains decreased in many chloroplasts in the leaves of cultivars ELSOMA and GS46 (Fig. 3 d). This was especially apparent in the case of GS46, where many of the chloroplasts lost their original bulging appearance due to large starch grains observed in all unstressed leaves. In leaves of cultivar ELSOMA, vesicular structures were present in some of the vacuoles and signs of plasmolysis could be observed. The granal and stromal thylakoids were still intact as were the chloroplast membranes (Fig. 3 d). Cells with granular vacuolar content were observed. Chloroplast membranes in leaves of GS46 were still intact and the thylakoids appeared normal in most of the cells investigated. Vesicular structures were observed in many of the cells. One of the most noticeable features of these cells was the granular or fibrillar material which collected in some of the vacuoles (Fig. 4). This was also discernible in the sections prepared for light microscopy and was not observed in any of the other cultivars at this stage.

After water had been withheld for 12 days ($\Psi_L = -2.51$ MPa), large lipidic globules, some more osmiophilic



Fig. 4: Distinctive water stress-induced appearance of granular or fibrillar material in the vacuole of cultivar GS46 after a Ψ_L of -1.97 MPa was reached. Bar = $1 \mu\text{m}$.

than others, appeared in chloroplasts of all the cultivars investigated (Fig. 3 e). Leaves of cultivar TL33 still contained starch grains in most of the chloroplasts (Fig. 3 e). The starch grains were smaller than those present in unstressed leaves. Cytosolic damage was very severe in some cells where vesicular material collected in large parts of the cell. Large osmiophilic accretions appeared in the degenerated cells. In severely damaged chloroplasts signs of bulging of the outer chloroplast membrane were observed. Some of the chloroplasts assumed a curved shape (Fig. 3 e) and the cells showed signs of plasmolysis. Leaves of cultivar CDL28 still contained cells with intact thylakoids. Some of the chloroplasts contained starch grains smaller than those observed in unstressed leaves, while other chloroplasts were almost or completely depleted of starch. This could also be observed by light microscopy. Chloroplast membranes were still intact. Fibrillar material collected in the vacuoles of some of the cells, where the tonoplasts appeared to be poorly defined or damaged. Vesicular structures were present in the vacuoles of some of the cells. Few signs of severe plasmolysis were detected. Leaves of cultivar ELSOMA contained chloroplasts with smaller starch grains (Fig. 3 f). Fibrillar or granular material collected in the vacuoles while few vesicular structures were observed in the vacuoles. Chloroplast envelope membranes were still intact, although the first signs of granal and especially stromal thylakoid dilatation were apparent in some of the cells. Signs of plasmolysis and curved chloroplasts were observed in some of the cells (Fig. 3 f). In leaves of cultivar GS46, the stromal and granal thylakoids appeared somewhat dilated but all membranes investigated were still intact. Small starch grains were present in the chloroplasts. Fibrillar or granular material was present in the vacuoles (Fig. 4). These cells showed little sign of plasmolysis.

Thus, although no complete loss of starch was observed at any stage during water stress, in contrast to the situation reported by Giles et al. (1976) in water stressed leaf tissue of *Sorghum bicolor*, a decline in the starch content was observed

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Pyrroline-5-Carboxylate Reductase Is in Pea (*Pisum sativum* L.) Leaf Chloroplasts¹

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ABSTRACT

Proline accumulation is a well-known response to water deficits in leaves. The primary cause of accumulation is proline synthesis. Δ^1 -Pyrroline-5-carboxylate reductase (PCR) catalyzes the final reaction of proline synthesis. To determine the subcellular location of PCR, protoplasts were made from leaves of *Pisum sativum* L., lysed, and fractionated by differential and Percoll density gradient centrifugation. PCR activity comigrated on the gradient with the activity of the chloroplast stromal marker NADPH-dependent triose phosphate dehydrogenase. We conclude that PCR is located in chloroplasts, and therefore that chloroplasts can synthesize proline. PCR activities from chloroplasts and etiolated shoots were compared. PCR activity from both extracts is stimulated at least twofold by 100 millimolar KCl or 10 millimolar MgCl₂. The pH profiles of PCR activity from both extracts reveal two separate optima at pH 6.5 and 7.5. Native isoelectric focusing gels of samples from etiolated tissue reveal a single band of PCR activity with a pI of 7.8.

Induction of proline accumulation by water deficit is a well-known, but little understood, phenomenon in plant stress physiology. Proline accumulation is caused primarily by increased synthesis from glutamic acid; the biosynthetic pathway is postulated to be analogous to that which converts glutamic acid to proline in *Escherichia coli* (21). The first two enzymes of this pathway have yet to be defined in plant extracts. The third and final enzyme PCR,² has been measured from several plant sources (12, 13), and Krueger *et al.* (10) have purified it to apparent homogeneity from wilted barley leaves.

The subcellular location of proline biosynthesis has not been clearly established. The involvement of light in this process has been indicated. Noguchi *et al.* (17) have shown that inhibition of PSII inhibits proline synthesis in tobacco leaf discs. Rajaopai *et al.* (20) have shown that the pattern of proline accumulation in drought-stressed wheat parallels the pattern of diurnal change in light intensity. PCR activity has been reported in chloroplast-enriched fractions from tobacco

leaves (16), but Kohl *et al.* (9) found that in soybean root nodules this enzyme is in the cytosol and not in plastids. To obtain an indication of the subcellular location of proline biosynthesis in leaves, we have investigated the subcellular location of PCR, the only proline biosynthetic enzyme for which a reliable assay in higher plants is available. Our results indicate that, like many other amino acid biosynthetic enzymes, PCR is located in the chloroplasts. We have also compared the properties of PCR from leaves and from etiolated shoots and find that they are similar.

MATERIALS AND METHODS

Plant Material

Peas (*Pisum sativum* L. var Argenteum) were grown in soil flats in a growth chamber under the following conditions: 16 h light, 8 h dark, 20°C, 270 $\mu\text{mol s}^{-1} \text{m}^{-2}$ combined fluorescent and incandescent light. Plants were watered with Hoagland solution (7) every third day and were allowed to wilt for 1 d each week. The wilting ensured that increased proline synthesis was stimulated. The Argenteum variety was used because its leaf epidermis is easily peeled away. Protoplasts were prepared from peeled leaves. Etiolated peas (*P. sativum* L. var Progress No. 9) were grown in coarse vermiculite at 30°C in darkness for 9 to 12 d and watered with deionized water. Progress No. 9 was used for experiments with etiolated tissue because sufficient Argenteum seed was not available.

Protoplast Preparation

Pea protoplasts were prepared from 4-week-old leaves of the Argenteum variety (24). Plants were destarched by placing them in darkness for 24 h. Adaxial epidermises were peeled and 40 leaves were floated on protoplast buffer (500 mM sorbitol, 5 mM Mes-KOH, 1 mM CaCl₂, [pH 6.0]) containing wall digesting enzymes (2% [w/v] Onozuka cellulase, 0.5% [2/v] Macerozyme, 1% [w/v] hemicellulase) and 0.2% (w/v) BSA in 8.5 cm Petri dishes in darkness at 30°C. After 1 h, the digestion medium was aspirated and 10 mL of protoplast buffer was added to each dish. Protoplasts were released by gentle rocking and decanted into a beaker, then another 10 mL of protoplast buffer was added, and the remaining protoplasts were decanted. Protoplasts were collected by centrifugation in a swinging bucket rotor at 100g_{max} for 1 min at 4°C. The supernatant was aspirated and discarded. Protoplasts were resuspended in 5 mL of chloroplast buffer (300 mM sorbitol, 1 mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA, 0.2% [w/v] BSA, 50 mM Mops-KOH [pH 7.2]).

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²Abbreviations: PCR, Δ^1 -pyrroline-5-carboxylate reductase; MMT, Mes, Mops, Tricine; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHAPSO, 3-[(3-cholamidopropyl)-diethylammonio]-2-hydroxy-1-propanesulfonate; GK, γ -glutamyl kinase; GPR, γ -glutamyl phosphate reductase; TPDH, triose phosphate dehydrogenase.

Protoplast Fractionation

Protoplasts were ruptured by passing the suspension three times through 20- μ m nylon mesh at the end of a syringe barrel. Microscopic examination revealed complete lysis of all protoplasts after this treatment. The resulting suspension was centrifuged in a Sorvall HB-4 swinging bucket rotor at 250g_{max} for 2 min. Pellets, which contained both damaged and intact chloroplasts, were resuspended in 3 mL chloroplast buffer and overlaid on a Percoll (Pharmacia) gradient. The gradient was generated by mixing 15 mL Percoll with 15 mL 2 \times chloroplast buffer in 50-mL polypropylene tubes and centrifuging at 40,000g_{max} in a Sorvall SS-34 fixed angle rotor for 30 min; the rotor was stopped without the brake. The overlaid gradient was centrifuged at 8,000g_{max} for 20 min in a Sorvall HB-4 swing-out rotor without the brake. A 12-cm-long, 20-gauge needle was inserted into the gradient to the bottom of the tube, and the gradient was fractionated into 1.6-mL aliquots using a peristaltic pump.

Extraction of PCR

Because Percoll interfered with enzyme assays, protein was precipitated from each Percoll gradient fraction by addition of 2.4 mL of a 50% (w/v) polyethylene glycol (3,500 average molecular mass) solution buffered with 50 mM Mops-KOH (pH 7.2). Samples were vortexed, incubated 10 min, and centrifuged in a Sorvall SM-24 fixed angle rotor at 20,000g_{max} for 10 min. Supernatants were aspirated and the pellets resuspended in 0.2 mL of 20 mM Tricine-KOH (pH 8.0), 5 mM MgCl₂, 10 mM β -mercaptoethanol, and 20% (v/v) glycerol.

Etiolated pea shoots were harvested and stored at -20°C. Five hundred g of shoots were homogenized in 500 mL grinding buffer (100 mM Mops-KOH, 1 mM EDTA, 20 mM MgCl₂, 10 mM β -mercaptoethanol, 5% [w/v] insoluble PVP) with a Polytron tissue homogenizer (Brinkmann Instruments) at 4°C for 5 min. The slurry was filtered through four layers of cheesecloth. After filtration, the extract was brought to 30% saturation with (NH₄)₂SO₄ at 40°C, incubated 15 min, and centrifuged in a Sorvall GSA rotor at 25,000g_{max} for 10 min. The supernatants were pooled, brought to 60% saturation with (NH₄)₂SO₄, incubated 15 min, and centrifuged again. The pellets were resuspended in 5 mL 20 mM Tricine-KOH (pH 8.0) and desalted on a Sephadex G-25 column (2.5 \times 28 cm) equilibrated with the same buffer. This preparation was made 20% (v/v) with glycerol and stored at -20°C.

For assays of crude chloroplast preparations, PCR was rapidly extracted from chloroplasts by the following modification of the procedure used for gradient purification of chloroplasts. The 250g pellet from a lysed protoplast suspension was resuspended in 6 mL of chloroplast buffer and recentrifuged at 250g for 2 min in a Sorvall HB-4 rotor. This pellet was resuspended in 2 mL of lysis buffer (20 mM Tricine [pH 8.0], 0.1 mM PMSF, 1 mM DTT) plus 1% (w/v) CHAPSO. After 10 min at 4°C, the sample was centrifuged in a Sorvall SS-34 rotor at 43,500g_{max} for 15 min at 4°C. The supernatant, which was yellow-green, was assayed.

Assays

The following assays were performed as previously described. NADP⁺-dependent triose phosphate dehydrogenase was used as a stromal marker (11), Chl as a thylakoid marker (11), Cyt c oxidase as a mitochondrial marker (1), and catalase as a peroxisome marker (2). The general PCR assay buffer contained 50 mM Tricine-KOH (pH 8.0), 1 mM DTT, 200 μ M NADH, and 2 mM D,L- Δ^1 -pyrroline-5-carboxylate (D,L-P5C). For the subcellular fractionation experiments the buffer also contained 100 mM KCl and 0.01% (w/v) Triton X-114. D,L-P5C was prepared by the method of Williams and Frank (26). P5C-dependent NADH oxidation was measured at 340 nm. The extinction coefficient of NADH (6.2 mm⁻¹ cm⁻¹) was used to calculate PCR activity. Kinetic parameters were determined by iterative fitting of the Michaelis-Menten equation (25).

Isoelectric Focusing

A modification of a previously described method (15) was used. Native isoelectric focusing was carried out in gels that were poured and run in a Mighty Small electrophoresis apparatus (Hoefer). Gels contained 4% (w/v) acrylamide, 0.0016% (w/v) methylene-bis-acrylamide, 1% (v/v) NP-40, 10% (v/v) glycerol, and 5% (v/v) Pharmalytes (pH 3-10). Gels were run at 200 V for 2 h followed by 400 V for 2 h. To locate PCR, gels were rocked for 2 h in 50 mL of 40 mM CAPS-HCl (pH 8.5), 100 mM L-proline, 1 mM NAD⁺, 1 mM Mg(OAc)₂, 100 mM KOAc, 300 μ g/mL nitroblue tetrazolium, 20 μ g/mL phenazine methosulfate. This staining system gives purple bands by the reverse (proline dehydrogenase) reaction of PCR, which is active at pH >9. Two-mm-thick gel slices were also assayed spectrophotometrically for PCR activity in the forward reaction. Slices were incubated in PCR assay buffer for 15 h. Reverse activity of the same samples were measured spectrophotometrically as previously described (12). In these assays forward PCR activity was fivefold greater than reverse activity.

RESULTS

Subcellular Localization of PCR Activity

A crude chloroplast suspension prepared from lysed protoplasts contained 25% of the PCR activity in the protoplast suspension (Table I). The suspension contained comparable proportions of two chloroplast markers, NADP⁺-TPDH activity (15%) and Chl (19%). This result suggests that PCR may be localized in plastids; however, the suspension also contained significant amounts of activity of a mitochondrial marker enzyme, Cyt c oxidase (6% of total activity), and a peroxisomal marker enzyme, catalase (13% of total activity). When this chloroplast-enriched preparation was fractionated by isopycnic Percoll density gradient centrifugation, the maximum PCR activity coincided with the maximum TPDH activity and Chl concentration (fraction 3, Fig. 1). There was almost no Cyt c oxidase activity in this region of the gradient, but some catalase activity was apparent. The proportion of catalase activity recovered in fraction 3 was only one-third the proportion of chloroplast markers and PCR recovered in

Table 1. Distribution of PCR and Markers in Subcellular Fractions from Pea Protoplasts

Marker	Total Units ^a			Percent of Total Along Gradient ^b	
	3 mL protoplast suspension	3 mL chloroplast suspension	Total along Percoll gradient ^c	Fraction No. 3	Fraction No. 17
PCR	0.8	0.20	1.02	35	9
TPDH	3.3	0.50	0.54	29	6
Chl	5.0	0.95	1.44	33	21
Cyt c oxidase	6.3	0.35	0.003	3	0
Catalase	4.2	0.55	0.14	11	17

^a 1 unit = 1 $\mu\text{mol min}^{-1}$ for enzymes and 1 mg for Chl. ^b Numbers in the third column represent 100%. ^c Sum of activities in all fractions.

that fraction (Table I). The lower recoveries of Cyt oxidase and catalase indicate that fraction 3 contained lesser proportions of mitochondria and peroxisomes than of chloroplasts. Moreover, the distribution of PCR activity in the gradient as a whole coincided more closely with the distribution of TPDH than with that of catalase or Cyt oxidase.

In three of seven replicate gradients (data not shown), PCR activity was prominent in the ruptured chloroplast region (fraction 17). This distribution suggests that PCR is associated with thylakoid membranes. Several nonionic detergents were added to the chloroplast lysis buffer to test their ability to dissociate interactions between PCR and thylakoids. The yield of PCR activity in the soluble fraction from chloroplasts was increased twofold by NP-40, CHAPS, or CHAPSO (data not shown).

Whole etiolated shoots yielded 100-fold greater PCR activity than chloroplasts from green leaves. When etioplasts were purified from pea shoots on a Percoll gradient, PCR activity ($42 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$) was detected, but the yield was less than 1% of the PCR activity of whole shoot extracts. The yield of etioplasts was itself very low: only 1.3% of the total NADP^+ -TPDH activity of etiolated shoots was present in the etioplast fraction ($102 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$). These results suggest that PCR is present in etioplasts, but do not provide conclusive evidence as to its subcellular distribution in etiolated shoots.

Kinetic Properties of PCR

The specificity of chloroplast PCR for pyridine nucleotide cofactors was investigated. At pH 7.5 and in the presence of 2 mM D,L-P4C, the enzyme had apparent K_m s for NADPH and NADH of 0.12 mM and 0.19, respectively, whereas V_{max} was greater with NADPH ($0.19 \mu\text{mol min}^{-1} \text{ mg protein}^{-1}$) than with NADH ($0.15 \mu\text{mol min}^{-1} \text{ mg protein}^{-1}$). The kinetic parameters of PCR from etiolated shoots were also examined. PCR from etiolated shoots had apparent K_m s for NADPH and NADH of 0.1 mM and 0.43 mM, respectively. The V_{max} was greater with NADH ($3.2 \mu\text{mol min}^{-1} \text{ mg protein}^{-1}$) than with NADPH ($0.83 \mu\text{mol min}^{-1}$).

There were two pH optima for PCR activity in chloroplast preparations, at pH 6.5 and at pH 7.5 (Fig. 2A). These optima are also present in PCR prepared from etiolated pea shoots (Fig. 2B) and similar optima were observed with both enzymes when NADPH was the substrate (data not shown).

Salt stress causes proline to accumulate (3, 8). Light increases the Mg^{2+} concentration in the stroma *in vivo* (19). The effects of salts on PCR activity were investigated to test the hypothesis that proline biosynthesis can respond to changes in ion concentration. One hundred mM KCl (Fig. 3) or 10 mM MgCl_2 (Fig. 4) caused an approximately twofold increase in the PCR activity of both chloroplast and etiolated preparations. Potassium acetate, NH_4Cl , and NH_4OAc stimulated PCR activity to the same extent as KCl (Table II). Maximal stimulation was observed at concentrations between 50 and 100 mM KCl. Activity declined at concentrations above 100 mM; at 200 mM KCl, activities were only slightly higher than the control activity of the chloroplast enzyme increased to a maximum at 10 mM MgCl_2 and did not differ between 10 and 20 mM (Fig. 4). Activity of the enzymes from

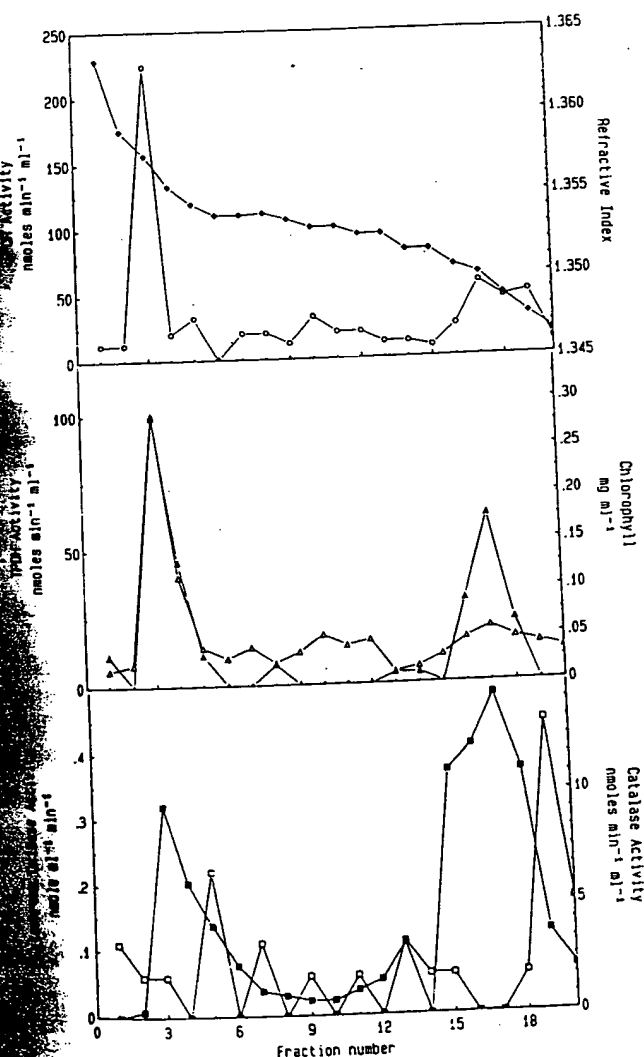


Fig. 1. Distribution of PCR activity and subcellular markers along a Percoll gradient: PCR (○), refractive index (◆), triose phosphate isomerase (Δ), Chl (▲), Cyt oxidase (□), and catalase (■). The samples contained 60 mg protein. Fraction 1 is the bottom of the gradient.

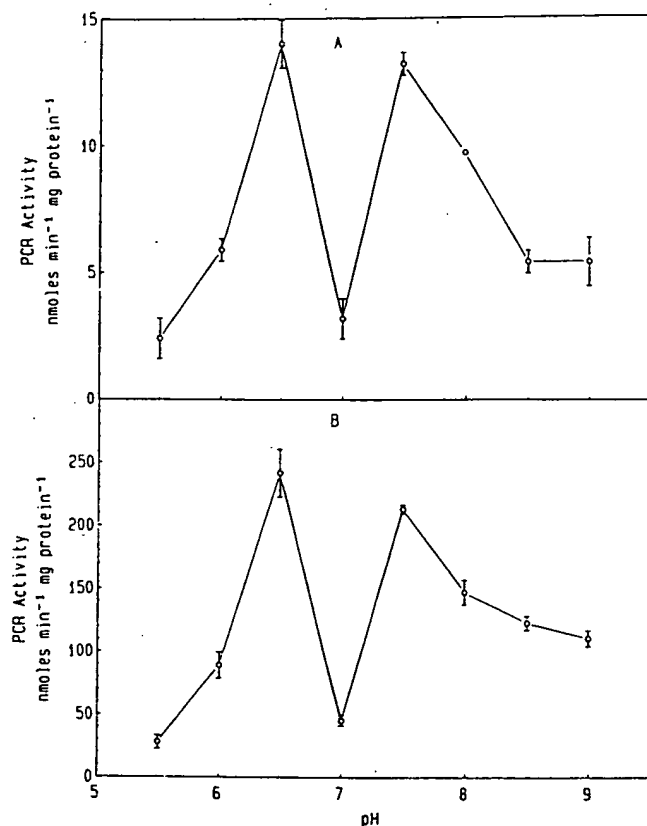


Figure 2. Effect of pH on PCR activity from pea chloroplasts (A) and etiolated pea shoots (B). MMT buffer used for the pH curve contained 50 mM Mes, 50 mM Mops, and 50 mM Tricine. HCl or KOH was used to adjust pH. Except for the change in buffer, the general PCR assay conditions were used, NADH being the substrate. Results are the means and standard errors of four assays.

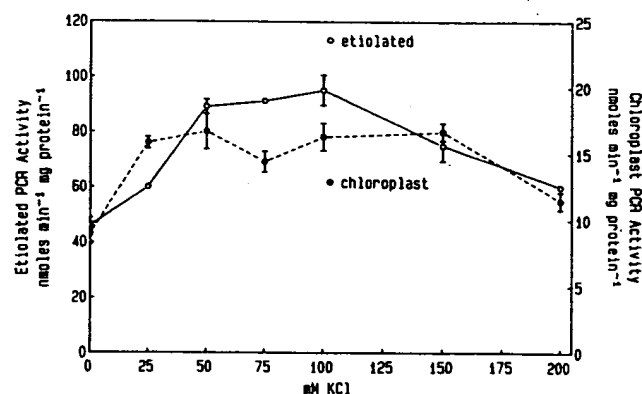


Figure 3. Effect of KCl on PCR activity from pea chloroplasts (●) and etiolated pea shoots (○). Assay was as described in "Materials and Methods," but KCl was added. Results are the means and standard errors of four assays.

etiolated tissue increased with increasing $MgCl_2$ concentration to a maximum at 5 mM, but activity at 10 and 20 mM was lower than it was at 5 mM (Fig. 4). Sucrose and sorbitol of osmolalities equal to that of 100 mM KCl did not cause any significant stimulation (Table II). Potassium acetate, ammonium chloride, and ammonium acetate stimulated PCR ac-

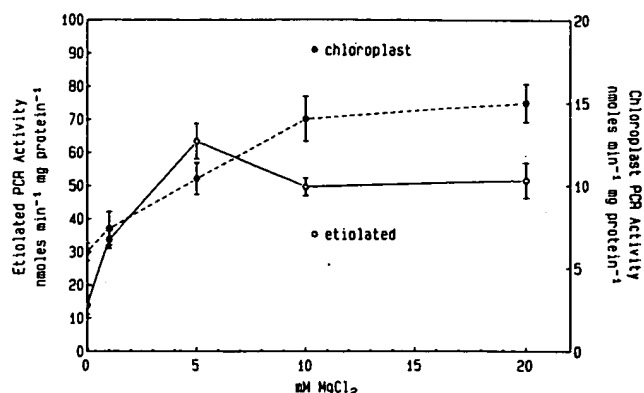


Figure 4. Effect of $MgCl_2$ on PCR activity from pea chloroplast (●) and etiolated pea shoots (○). Assay was described in "Materials and Methods," but $MgCl_2$ was added. Results are the means and standard errors of four assays.

Table II. *In Vitro* Effects of Salts and Sugars on PCR Activities^a from Pea Leaf Chloroplasts and Etiolated Pea Shoots

Treatment	Chloroplast	Etiolated Shoot
	nmol min ⁻¹ mg protein ⁻¹ ^a	
Control ^b	4 ^c (1.0)	35 (7)
Monovalent Cations		
+100 mM KCl	9 (0.2)	138 (31)
+100 mM KOAc	9 (1.1)	127 (3)
+100 mM NH_4Cl	10 (0.6)	162 (3)
+100 mM NH_4OAc	8 (1.2)	142 (39)
+200 mM Sucrose	3 (0.5)	39 (5)
+200 mM Sorbitol	3 (0.6)	39 (4)
Divalent cations		
+10 mM $MgCl_2$	13 (0.7)	132 (19)
+10 mM $MnCl_2$	14 (1.3)	170 (10)
+10 mM $CaCl_2$	13 (1.3)	164 (23)
+10 mM $Mg(OAc)_2$	14 (0.7)	117 (33)
Combined cations		
+10 mM $MgCl_2$ + 100 mM KCl	8 (1.0)	147 (8)

^a Mean (SE). Each value represents the mean of four assays.

^b Assay described in "Materials and Methods."

tivity to the same extent as KCl (Table II). Manganese chloride, calcium chloride, and magnesium acetate caused the same stimulation as magnesium chloride (Table II). The effects of KCl and $MgCl_2$ were not additive at concentrations that gave maximal stimulation alone (Table II).

Isoelectric Focusing

Separation of the pH 6.5 and pH 7.5 PCR activities from etiolated shoots was attempted by native isoelectric focusing. One zone of activity ($R_f = 0.44$, pI 7.8) contained PCR activity (Fig. 5). Activity of the reverse PCR reaction (P5C dehydrogenase activity, pH 9.5) produced bands of purple precipitate in the same region. The results were the same when NADP⁺ was the substrate.

DISCUSSION

Noguchi *et al.* (16) described the localization of PCR activity in a chloroplast-enriched fraction from tobacco leaves.

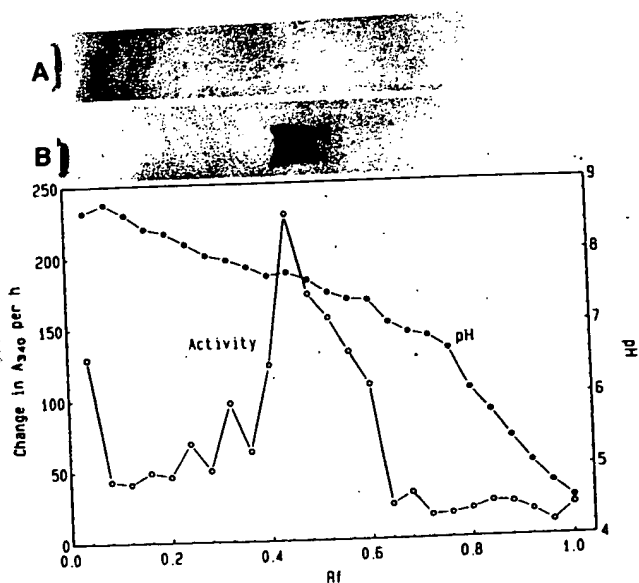


Figure 5. PCR activity (○) and pH (●) in 2 mm segments of a native isoelectric focusing gel. R_f = position of proteins in segment relative to position of a pl marker, methyl red. The general PCR assay was used with the addition of 100 mM KCl. This gel was stained for proline dehydrogenase activity (pH 9.5), (A) without proline, and (B) with proline.

Ohl *et al.* (9), however, found that in soybean nodules, PCR was localized in the cytosol and not in plastids. The results in Figure 1 and Table I demonstrate that in green pea leaves PCR is present in chloroplasts, since the distribution of PCR activity when leaf protoplasts are fractionated most closely resembles the distribution of the stromal marker NADP⁺-triose phosphate dehydrogenase. Most significantly, PCR activity was most abundant in the same fraction of Percoll gradients as TPDH. About 25% of the PCR activity measured in protoplast suspensions (Table I, column 1) was recovered in chloroplast suspensions (Table I, column 2), while the recovery of intact chloroplasts from protoplasts was approximately 15 to 20% based upon the recovery of NADP⁺-TPDH and Chl. Because the chloroplast-to-protoplast ratio for PCR activity is greater than the same ratios for chloroplast markers, it appears that PCR in plastids can account for all of the pea PCR activity. This distribution contrasts with the localization of PCR activity in the cytosol of soybean root nodules (9). PCR from pea leaf chloroplasts uses both NADH and NADPH as electron donors, but has a lower K_m and higher V_{max} for NADPH. Thus, like PCR from barley (10) and tobacco leaves (16), it is more active with NADPH. This specificity is consistent with the chloroplast localization of this enzyme, although the enzyme from soybean root nodules is also more active with NADPH except at very high cofactor concentrations (9).

Preliminary evidence indicates that PCR is present in etiolated shoots from etiolated pea shoots. Although PCR from etiolated shoots has a lower K_m for NADPH than for NADH, its V_{max} /ratio with the two cofactors indicates that it is more active with NADH than with NADPH. Thus, the most abundant form of the enzyme in etiolated shoots may be different from that in green leaves.

The bimodal pH curve for pea PCR is analogous to that reported previously for PCR in extracts from etiolated barley (*Hordeum vulgare*) and etiolated mung bean (*Vigna radiata*), both of which give broad pH-activity curves with optima or shoulders at pH 6.4 and 8.0 (4). The bimodal pH curve was also observed when NADPH was used as the cofactor. The pH 7 minimum is not an artifact, because the pH profile was reproducible with batches of MMT buffer prepared three different times. The bimodal curve could be produced by a single enzyme or two isoforms. If there are two forms in green leaves, both are probably present in chloroplasts. The ratio of the pH 6.5 to pH 7.5 activities in chloroplast-enriched preparations was equal to the ratio in whole etiolated shoot extracts. PCR in whole protoplast extracts from green leaves produced a broad pH curve. Activities at pH 6.5 and 7.5 were similar (data not shown), as in the chloroplast-enriched fraction and in etiolated shoots. There was no minimum apparent at pH 7.0, but the enzyme was difficult to assay precisely in whole protoplast extracts because of its low activity. This investigation focused on the PCR activity with the pH 7.5 optimum.

The native isoelectric focusing gel revealed only one region of PCR activity, with a pI of 7.8; the same pattern of PCR activity was detected with NADH and NADPH. Thus, it appears that if there are to isoenzymes in etiolated shoots, they have the same pI. An alternative explanation is that one of the isoenzymes is inactivated during native isoelectric focusing. This activity does not represent the activity of mitochondrial proline dehydrogenase: the mitochondrial proline dehydrogenase is not active at pH 9.5 (4), and it does not donate electrons to NAD⁺. The genetic basis for the pH dependence of PCR activity remains uncertain.

The stimulation of PCR by salts is unclear but it appears to be a function of ionic charge. Because potassium ion concentrations in chloroplasts are normally around 100 mM, the enzyme should be fully active *in vivo*. Changes in stromal pH between light and dark occur in the range that includes the pH optima observed in this work. Stromal pH in the dark is approximately 7, corresponding to the trough in activity; stromal pH approaches 8 in the light, so that the pH 7.5 activity would function in the light. Proline synthesis occurs in leaves in both light and darkness (6, 16, 17, 23), but is stimulated in light.

A role for proline as a redox shuttle molecule has been established for some mammalian tissues (20) and has been proposed for nitrogen fixing nodules (9). The existence of such a shuttle mechanism in leaves needs to be investigated. If such a shuttle operates in leaves under nonstressed conditions, then the localization of PCR in chloroplasts would function to transport reducing potential to mitochondria in the form of proline. To test such a redox shuttle hypothesis, a mechanism that transports proline across the chloroplast envelope needs to be identified. An uncoupled redox shuttle mechanism would contribute to proline accumulation. If water deficit caused such a shuttle to become uncoupled by decreasing proline oxidation without decreasing proline synthesis, then proline accumulation could be a symptom of metabolic dysfunction. Because proline has biocompatible characteristics and proline accumulation is not toxic, metab-

olism may be temporarily shunted in this direction until homeostasis is regained.

Since the product of P5C reductase is proline, the results of this investigation provide evidence that, like many other amino acids, proline is synthesized in chloroplasts. It will be of considerable interest to determine whether the enzymes catalyzing conversion of glutamate to P5C have the same subcellular location.

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